CONTRIBUTIONS FROM THE CRYPTOGAMIC LABORATORY OF HARVARD UNIVERSITY. — LVIII.

SEXUAL REPRODUCTION IN THE MUCORINEAE.

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PREFACE.

For several years past the writer has been engaged in a study of the Mucorineae, which he at first intended should comprise a general survey of the morphology and development of the group, and include at least an attempt to set in order some of the confusion in which the systematic study of these plants has been involved. Having accumulated a considerable amount of material for the purpose, the greater part of which has been kept under cultivation in pure cultures, he was led about a year since to abandon his original plan and restrict his field of investigation to a single phase of the subject in hand, and to confine his attention to the conditions influencing, or associated with, the production of the so-called "sexual spores," or zygospores, which are characteristic of these fungi. Although during the past thirty or more years this subject has received much attention from botanists, and has been the object of numerous researches, the results obtained in the present instance appear to be quite novel, and, in the writer's opinion, present for the first time a rational basis by means of which the phenomena of sexual reproduction in the group may be explained.

That the present paper, which is practically based on hardly a year's work, embodies a necessarily inadequate treatment of the subject need hardly be pointed out; but although the necessities of the case have rendered this incomplete presentation unavoidable the writer proposes to continue his investigations, and trusts that he may be able in a future communication to remedy in some measure the deficiencies of the present one.

It is a pleasurable duty to acknowledge at this place indebtedness to those who have kindly given assistance in one way or another: to Dr. W. G. Farlow for the use of his herbarium and for several of the papers

herein cited; to Prof. E. C. Jeffrey for photographing certain of the plates; but above all, to Prof. R. Thaxter, who, besides being the one under whom this research has been prosecuted and to whom a pupil's thanks are due for advice and encouragement, has placed at the writer's disposal his mounted specimens and tube cultures of forms of the Mucorineae which have been accumulated during his extensive investigations among the lower fungi.

Thanks are due to the following for material: Prof. George Klebs; Prof. H. M. Ward, and Dr. R. H. Biffen; Prof. O. Brefeld, and Dr. R. Falck; Prof. D. H. Campbell; Prof. F. Cavara; Prof. Ed. Fischer; Prof. P. Vuillemin; Mr. Robert M. Grey; Mr. C. S. Leavenworth; Mr. J. B. Rorer; Mr. J. J. Wolfe; and to numerous others both in America and Europe for similar favors, especially in connection with the Rhizopus table (p. 263).

Contents.

PART I.

INTRODUCTION.

Among the Mucorineae the usual form of reproduction is by means of nonsexual spores in sporangia. Although the sexual method of reproduction by zygospores has, through the researches of individual investigators, now been found in nearly all the genera, it is still in a majority of the species entirely unknown, and our knowledge of its occurrence in about four-fifths the cases rests on the unconfirmed reports of single individuals. The only common species which could be depended on to produce its zygospores has been *Sporodinia grandis*. Ever since de Bary's ('64) investigation of this form and of *Rhizopus nigricans* ('66) the causes inducing the formation of zygospores have been a subject of much speculation and considerable study among mycologists. The elaborate papers of Klebs ('98) and Falck ('01) have given us much conflicting information on the problem in Sporodinia, but attempts to apply the results of these investigations, or of the earlier researches of van Tieghem and others, to the Mucorineae in general, with a view to obtaining zygospores, have proved ineffective. Since the evidence on this subject, in a form like Sporodinia for example, which has been most thoroughly examined, is not only conflicting, but is evidently inapplicable to a majority of existing forms, it has been the aim of the present paper to consider the conditions which lead to the production of zygospores in the group as a whole rather than in any single species.

In 1901, at Professor Thaxter's suggestion and under his direction, the writer began the collection and preservation in pure cultures of forms of the Mucorineae, but especially of the genus Mucor, having in mind, as has already been mentioned, a subsequent monograph of this confused genus. In the course of this preliminary work zygospores of different species were several times found, but the forms differed greatly in their behavior. Some of them, among which Sporodinia may be taken as a type, could be readily induced to produce zygospores on a suitable substratum when a pure transfer was made by means of spores taken from a single sporangium; others, of which Rhizopus may be taken as a type, would never form zygospores from a pure transfer, but only when a mass of spores from a zygosporic culture was used for the inoculation. Of the first class, all have been isolated and kept running in tube cultures without abatement in their zygosporic activity, but with such species of the second as could not be separated from the abundance of other fungi and bacteria and preserved with their zygospores on the dried substratum, it

was impossible to continue the formation of zygospores. In the attempt to grow a form of the Rhizopus type on nutrient agar in a more convenient condition for sectioning, a separation culture was made from the midst of the zygosporic region, and zygospores were found (May 2, '03) to appear at the junction of certain of the mycelial colonies. By following up this suggestion it was demonstrated that this species consists of two strains or races which, when grown apart, produce only sporangia, but which form zygospores when hyphae from the physiologically different mycelia are allowed to come in contact. If we designate one strain by the sign (+) we can conveniently use the (−) sign to represent the other without committing ourselves as to the sexual relation which the strains may bear to each other. A (+) strain, for example, will never produce zygospores on any medium if sown alone, nor if sown with some other (+) strain however different in origin; but if the complementary (−) strain be sown at the same time either mixed with the (+) or so disposed that their mycelia can come in contact, zygospores are formed by the union of gametes produced from the (+) and (−) mycelia respectively. The condition is essentially similar to that in dioecious plants and animals, and although in this case the two complementary individuals which are needed for sexual reproduction are not in general so conspicuously differentiated morphologically as in the higher forms, such a morphological difference is often distinctly visible, and, as hereafter noted, would undoubtedly be considered by systematists generally as an amply sufficient basis for their specific separation. Inasmuch, however, as conjugation is possible only through the interaction of two differing thalli, we can express this fact by calling all species the sexual relations of which correspond to the Rhizopus type, *heterothallic*. In marked contrast to the conditions just described, Sporodinia and the other members of the group of which it is the type, invariably reproduce sexually under suitable conditions when grown from a single spore. The zygospores thus originate from a single mycelium, and are comparable to hermaphrodites among the higher plants. Such forms may therefore be called *homothallic*.

In several of the heterothallic forms experimented with, certain races have been found which apparently cannot be induced to respond to the (+) and (−) strains, the existence of which has been demonstrated in such species. Moreover it has been shown that in *Mucor Mucedo* the power of conjugating possessed by the two sexual strains may be at will entirely inhibited by cultivation under unfavorable conditions. These "neutral" strains will be further discussed in connection with the individual forms considered in Part II.

It may be here stated that the writer has at present under cultivation over a dozen forms, including both types, which will conjugate whenever the proper conditions are furnished. Of the heterothallic group five species of the genus Mucor are represented, including *M. Mucedo*, besides *Rhizopus nigricans*, *Phycomyces nitens*, *Absidia caerulea*, and an undescribed species of a new genus. Of the homothallic are *Sporodinia grandis*, *Zygorhynchus Moelleri*, *Dicranophora sp.*, and two undescribed species of Mucor. The undetermined Mucors will be provisionally indicated by Roman numerals, Mucors I and II being homothallic, Mucors III to VI heterothallic, while Mucor N represents the undescribed new genus.

It is the purpose of the writer to give in Part I a brief historical review of the theories that have hitherto been advanced in regard to the causes influencing zygospore formation, followed by a citation as complete as the writer has been able to make it, of all the species of which this form of reproduction has been reported. In Part II the original research on the subject of this paper will be arranged under the different species investigated.

In this connection it may be advisable to define certain terms, some of which have been used variously by different authors, and to give a short account of the morphology of conjugation. In Rhizopus or in Sporodinia, for example, wherever two adjacent (+) and (−) hyphae touch, the stimulus of contact may cause outgrowths to form; which, by remaining mutually adherent, and increasing in size, gradually push apart the hyphae from which they have arisen. These outgrowths — the gametes of some authors (e. g. Léger, '96) — may better be termed *progametes*, since each becomes divided by a septum into a proximal portion, the suspensor, and a terminal cell, the gamete proper, and it is by the union of these gametes that the zygote is formed. This terminology brings the process into accord with other plants and with animals. Here, instead of dividing and developing into an embryo, the zygote becomes transformed into a single-celled, thick-walled, resting zygospore. It sometimes happens that, owing to unfavorable conditions, the gametes cut off fail to unite and develop independently into thick-walled azygospores. The formation of these spores may be considered abnormal, but in certain species where conjugation is not known to occur, so-called azygospores have been found to be produced regularly in great abundance. These reproductive bodies have been considered in connection with the zygospores in the literature cited, but it is impossible to state at the present time what their significance can be.

Progametes may either arise from undifferentiated aerial hyphae, as in Rhizopus, or may originate as in *M. Mucedo*, from special branches, which, rising from the mycelium, are mutually attracted and develop their progametes at the point of contact. It will thus be convenient to distinguish two types of more or less distinctly differentiated fertile hyphae; namely, sporangiophores bearing nonsexual spores in sporangia, and *zygophores*, which give rise to progametes and zygospores, although this distinction is not always well marked and both types of spores may originate from the same hypha. The successive steps in the process of conjugation as it occurs in *Mucor Mucedo* is represented in Plate II, Figures 25–35.

With the exception of de Bary's ('66) early description of Rhizopus, which is not in accordance with his later statements ('84, p. 159), and Falck's ('01, p. 241) description of Sporodinia, which, however, he was unable to confirm by observation, writers on the Mucorineae, in describing the process of conjugation, have stated that club-shaped progametes develop from opposite sides of hyphae not already in contact, and, increasing in size, grow through mutual attraction to meet each other at their swollen extremities. This account, illustrated with figures, has been generally current in text-books which mention the subject. However, in the species in which the process has been observed by the writer, including both homo- and hetero-thallic forms, the progametes are from the very first always normally adherent and arise as the result of the stimulus of contact between the (+) and (−) zygophoric hyphae which in some cases at least have been shown to be mutually attractive, and may therefore be termed *zygotactic*.

Although there is in general little if any differentiation in the conjugating apparatus which might possibly indicate a sexual differentiation, yet two genera of the homothallic group — Zygorhynchus and Dicranophora — are *heterogamic* in that their gametes show a certain constant inequality in size which corresponds to even a greater difference in size and form of their suspensors and zygophores. All other species of this group known are apparently isogamous.

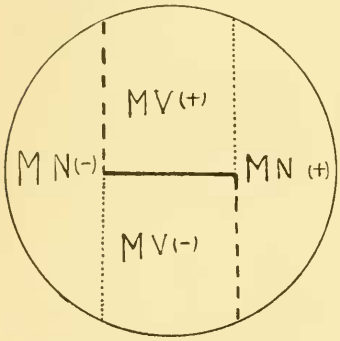
In the heterothallic forms, where we should perhaps most expect to find a structural difference in the conjugating cells, the gametes are morphologically equivalent, so far as has been determined. Differences in the size of the gametes or of the suspensors, as, for example, in Rhizopus, and priority of one suspensor over the other in developing outgrowths, as in Phycomyces, although considered by some authors as indicative of a sexual differentiation, are inconstant, and will be subse-

quently shown to have no sexual significance. It is true in nearly every heterothallic species investigated that the strains show certain morphological differences which correspond to their physiological differentiation as sexual strains. In general the difference is one in degree rather than in form, and is indicated in several ways. *Mucor Mucedo* may be taken as an illustration of the simplest type of such a differentiation from the fact that, although the two strains are not distinguished by any apparent morphological difference, the (−) strain has been shown to lose its sexual capacity sooner than the (+) strain. This difference is further indicated by the fact that, while successive mycelial transfers from young cultures of the (+) strain retain their sexual capacity through indefinite generations, this, with the power of vegetative development, is rapidly lost by the (−) strain when subjected to a similar treatment. In Phycomyces the differentiation is more distinct in that the sporangiophores are more numerous and less delicate, and develop later in one strain than in the other (on proper nutrients this is more marked than in the culture photographed, Plate IV, Figure 52), while in several species in which a still greater differentiation is exhibited, when the strains are contrasted one will regularly show a lower, less luxuriant, sporangial growth, and often a decided color contrast. The most striking instance of this kind is seen in the species photographed (Plate IV, Figure 58), where the difference in luxuriance of sporangial growth, added to a corresponding difference in the size of the spores (Plate I, Figure 23, 24), might easily lead one in investigating these strains separately to consider them as individual species.

It is thus evident from even a cursory examination of the known heterothallic species that their sexual strains are with few exceptions characterized by more or less marked differences, especially in relation to their relative luxuriance of growth, and it was owing to this peculiarity that it was thought appropriate to apply the terms (+) and (−) to the more and less luxuriant strains respectively. The assumption that their differentiation to (+) and (−) strains was indicative of a corresponding sexual difference, and that the strains thus designated should be regarded respectively as male and female, or vice versa, although naturally suggested by the observed condition, did not at first appear to be warranted. Evidence, however, is not lacking which would seem to justify such a conclusion. Plate IV, Figure 57, for example, shows the strains of Mucor v, forming zygospores at their median line of contact. On either side of these conjugating strains were sown corresponding strains of Mucor N (+) at right, (−) at left. Where the growth of the (+) strain of one

species comes in contact with the (—) of the other, a white line will be observed which a microscopic examination has shown is composed of incipient stages of conjugation wherein abundant progametes, and less frequently gametes, have been formed. It is an important fact that only when strains having unlike signs come in contact do these attempts at hybridization occur.

The accompanying diagram will perhaps render clearer the condition shown in the culture photographed. The continuous line between Mv(+) and Mv(—) indicates the zygospores formed by the two sexual strains of this species. The broken lines between Mv(+) and Mn(—), and between Mv(—) and Mn(+), indicate the lines formed by the attempts at hybridization between those strains of the two species which correspond to different signs. The dotted lines indicate the contacts of those strains of the two species corresponding to like signs. No distinct line is observed here in the photograph, and a microscopic examination shows that no attempts at hybridization have occurred.



By making contrasts between the (+) and (—) strains of those heterothallic species which have been producing zygospores in the laboratory it has been possible to arrange in a series all strains which had been marked (+) on account of their vegetative characters, and in another series all those marked (—) on the same basis. Those in the (+) series are found to be capable of forming imperfect hybrids with those of the (—) series, but no attempt at conjugation can ever be obtained between strains with like signs, either in the same or in different species.

It is further possible to place in the proper sexual columns the strains of those species (e. g. Rhizopus) in which a vegetative differentiation has not yet been determined (cf. table, p. 305), by means of the tendency already referred to, which leads to attempts at hybridization when (+) and (—) strains of different species, and even genera, are made to grow in contact with one another. Such an attempt at hybridization between Absidia and Rhizopus, for example (Plate I, Figure 18), shows in the most advanced cases the characteristic appendages from the Absidia suspensor,

and the hyphae of these forms as in many other species differ sufficiently to be distinguished even without tracing them to their sporangia. Perfect hybrids have never been found, and though for convenience the term hybridization will be used hereafter in the present paper, it should be remembered that these attempted imperfect conjugations have never been observed to go further than, at most, a cutting off of the firmly united gametes.

In other organisms, where the sexes are segregated on separate individuals, the female is generally characterized by a greater vegetative development, but the assumption that the same condition prevails here, and (+) and (−) strains are equivalent to female and male, respectively, would from our present knowledge hardly seem justifiable. It is hoped, however, that further investigation may demonstrate the correctness of such a conclusion.

A further indication of the strictly sexual nature of these processes in general is found in the interaction between homothallic forms which may be assumed to be bisexual [(+) *and* (−)], and (+) and (−) strains of heterothallic types. Plate IV, Figure 56, for example is photographed from a culture in which the (+) and (−) strains of a heterothallic species, Mucor v, have been sown on opposite sides of a homothallic species, Mucor i. Had a (+) or (−) strain of a heterothallic species been sown in place of Mucor i, conjugation would have taken place on one side only, namely, on that side where (+) and (−) strains were opposed. In the present instance, however, the middle strain having both (+) and (−) qualities, conjugates with the (+) heterothallic strain on the one hand, and with the (−) strain on the other, as is indicated by the evident white lines of demarcation between them. The response here demonstrated between a homothallic form and *both* (+) and (−) heterothallic strains which have been shown to be unisexual, can thus be explained only on the assumption that the homothallic Mucor i possesses both sex qualities.

It may be mentioned in this connection also that a number of species the zygospores of which are unknown have been made to produce imperfect hybrids with the (+) and (−) strains under cultivation. Thus individual strains of Syncephalastrum, a new species of Chaetocladium, and a number of other forms, have been found to be (−) from the fact that they interact only with (+) strains, while a culture of *Cunninghamella echinulata* Thaxter (*C. Africana* Matruchot), which was formerly classed as a Hyphomycete, has been shown to be (+). From our present knowledge one is justified in assuming that a culture which responds sexually to only one of the test strains must belong to the heterothallic group,

and its zygospores have not been seen simply because it has not been found in contact with its complementary strain. On this basis we can add to the heterothallic forms already known representatives of the genera Syncephalastrum, Circinella and Chaetocladium, and this will extend heterothallism to all the families of the Mucorineae recognized by A. Fischer ('92) except the Mortierellaceae. No attempt has yet been made to determine the sexual condition in this family.

From the fact that the writer has demonstrated the possibility of running out the sexual activity in *Mucor Mucedo,* and the apparent impossibility of obtaining zygospores from contrasts made between certain races of *Rhizopus nigricans* and (+) and (—) strains of this same species, it is further evident that neutral strains, or at least strains in which the sexual activity is dormant, exist in nature. The character of the substratum, and also the nature of the forms contrasted, are important factors to be considered in hybridizing. Phycomyces, for example, will hybridize with *Mucor Mucedo* only on concentrated nutrients, but will not hybridize with certain other forms under the same conditions. Consequently a failure to obtain imperfect hybrids by contrasting a given culture with the opposite strains of a test form proves nothing positive in regard to the thallic condition of the culture under investigation.

HISTORICAL REVIEW OF THE THEORIES RELATING TO ZYGOSPORIC REPRODUCTION IN THE MUCORINEAE.

With the foregoing brief review of the more important matters treated in the present research, and before considering them in detail, it has seemed desirable to present a short summary of the more prominent views which have been held by previous investigators regarding the conditions influencing the formation of zygospores in the Mucorineae, and the nature of the processes involved, to which is appended as complete a statement of the reported occurrence of these bodies as it has been possible for the writer to compile.

In Ehrenberg's (['20]'29) work on Syzygites, which contains the first mention of the occurrence of zygospores among the Mucorineae, he recognizes the conjugation as comparable with the act in such algal forms as Spirogyra and as of a probable sexual nature. Subsequent investigators, however, who have advanced theories to account for the appearance of zygospores, either deny that there is any sexuality in the process of their formation, or consider such a possibility as without bearing on the problem. Such explanations as have been offered are thus based in general on the assumption that zygospore formation is primarily depend-

ent on external conditions in connection with which they have been developed.

We are indebted to de Bary ('66) for the first discussion of this question, in his investigation of the common heterothallic form *Rhizopus nigricans*. Zygospores were found to form abundantly in tightly closed cultures, but less abundantly and only in the lower parts of cultures open to the air. "The fungus," according to this author, "takes in oxygen and gives off carbon dioxide; therefore in a closed tube, and below the network of hyphae in a gross culture the oxygen content of the air must be reduced. This circumstance, in that it retards the process of oxydation in the fungus, is apparently influential in the formation of zygospores, since the composition of the substratum, the water-supply, and the temperature, were the same in the open as in the closed cultures." In direct opposition to this conclusion, it may here be mentioned that Bainier ('83b, p. 342) gives the exclusive presence of oxygen as a cause previously assigned for the production of zygospores, but the writer has been unable to discover who is responsible for this theory.

By following de Bary's suggestion, van Tieghem ('75) obtained the zygospores of Rhizopus, and appeared to confirm his theory by his results with other forms. In his Troisième Mémoire (p. 324) he concludes: "That which determines the formation of zygospores upon a mycelium still provided with a sufficient quantity of protoplasm is the impoverishment of the nutritive medium in one or more of its elements, an impoverishment which places in danger even the existence of the plant." The total nutrient he divides into (1) air, or its oxygen at the pressure of one-fifth of an atmosphere; (2) water; (3) the soluble substances, or food proper. Reduction in oxygen is the cause assigned for the occurrence of zygospores in Sporodinia and Rhizopus; desiccation for the occurrence of those found by Cornu in *Absidia septata;* while for the appearance of the zygospores of Piptocephalis and Sporodinia obtained by Brefeld on bread soaked with beer wort, the cause is supposed to be the unfavorable condition of the food supply of these naturally parasitic plants. Although van Tieghem adds that he gives the above generalization with great reserve, since it is necessary to determine in each case the special nature of the impoverishment which is effective, yet his last word on the subject ('91, p. 1076) is a reiteration of his impoverishment theory with the mention of cold as an additional factor.

From his experience in obtaining zygospores of *Absidia septata*, Sporodinia and Spinellus, Cornu ('76) concludes, "It is when the substratum becomes more and more dry that the zygospores appear."

Bainier ('82, '83[b], '84, '89, '03) has been especially successful in obtaining zygospores, but though he concludes that the substratum has much to do with their production, he is at variance with previous authors in that he says ('83[b], p. 344) "when the plant finds the nutriment inferior it produces sporangia; when the food supply is abundant, zygospores appear." He looks upon the zygospores as so much stored-up nutriment, and consequently implying a very vigorous condition in the plant producing them, and demanding that the thallus be able to extend itself in length and depth. His point of view, with the theory resulting from it, is thus radically different from that of van Tieghem, who looked more on the function than on the formation of this condensed mass of nourishment, and consequently considered that the formation of a resting spore must be brought about by the appearance of those unfavorable conditions against which it was designed to protect the plant. As regards moisture, Bainier thinks there can be nothing said in general, the species differing in their preference for more or less dryness. The season of the year and the temperature seem to be with him factors of some importance, for in his notes on the zygospores of different species the months in which they are supposed to form is generally stated, and in his latest work ('03), where zygospores are described for a considerable number of species, the special condition mentioned is the low temperature of a cellar where his cultures were kept during a warm period of summer. A majority of Bainier's zygospores were obtained in van Tieghem cell cultures in a concentrated decoction of plum and prune, which was sterilized by boiling and mixed with ten to twenty per cent of alcohol to prevent fermentation. This investigator further states that freedom from parasites is a condition to be satisfied before zygospores can be expected, in marked contrast to the opinion expressed by Zopf ('88 and '92) in connection with his study of Pilobolus, where the suppression of sporangia through the attacks of a parasite is regarded as responsible for the appearance of zygospores (cf. p. 238 of present paper).

Brefeld's ('75, '81, '00) position has been one of agnosticism as regards the causes for the appearance of zygospores, and the valuable part of his conclusions is contained in a series of denials substantiated by the results of his cultures. The character of the nutriment alone he holds to be of slight significance, since on the same substratum from differently derived spores of the same fungus, e. g. Piptocephalis, he obtained on the one hand zygospores, and on the other only nonsexual spores. Changing the substratum and the oxygen content of the air did not alter the results. The age of the mycelium is likewise not considered influen-

tial, since he has cultivated a mycelium for four weeks on an unlimited substratum with only nonsexual reproduction. The sexual reproduction is not necessarily induced after a series of nonsexual generations, since he has cultivated Rhizopus, *Mucor Mucedo*, and *M. racemosus* for four years up to nearly one hundred nonsexual generations. With Sporodinia and Spinellus no culture fails to show zygospores; with *Mortierella Rostafinskii*, Piptocephalis, and Chaetocladium, zygospores form after a few generations of nonsexual reproduction; while with Rhizopus and *M. Mucedo* a longer time elapses before the appearance of zygospores. There is thus, according to Brefeld, a gradual withdrawal of the sexual in favor of the nonsexual reproduction, till in such forms as *M. racemosus* and Pilobolus this has resulted almost or entirely in the disappearance of sexuality. Brefeld's ('00 and '01) final conclusion is that in most Zygomycetes the zygospore formation is dependent on unknown inner causes, and it is only by accident that it may be found.

While the authors thus far cited have based their theories and conclusions chiefly or wholly on what they could learn from the chance occurrence of zygospores in their cultures, and scarcely ever resorted to experiments, more recent investigators have endeavored to subject their processes to a more exact experimental research. Klebs ('98) was the first to investigate in detail the effect of varying external conditions on the resulting form of reproduction of any of the Mucorineae, and in his paper on Sporodinia we have a careful account of the factors influencing sporangium and zygospore formation in this homothallic form. Van Tieghem's idea that lack of oxygen is the cause for zygospore formation is disproved, and it is shown that all the influences which hinder sporangium formation, as a reduction in the amount or in the concentration of the nutrient, hinder much more the formation of zygospores. Most of the carbohydrates tested with gelatine and the acid salts of organic acids favor zygospores, while the nitrogen compounds are unfavorable. The immediate cause of sporangium formation is held to be transpiration from the hyphae, and though impoverishment of nutriment has a similar effect it is impossible to obtain zygospores in relatively dry air. The stimulus for production of zygospores, however, is conceived to be the decreased transpiration due to increased relative humidity in the air. The limits of concentration within which zygospores are produced vary greatly, but for grape sugar in gelatine, lie between one per cent and fifty per cent. The water content of the substratum, temperature, and light, are effective only as they influence the relative humidity and consequent transpiration. The formation of azygospores is said to be induced in

a culture beginning to form zygospores by a change to any one of a number of unfavorable conditions.

In marked contrast to the results of Klebs, Falck ('01) finds that, within normal limits, relative humidity of the air and transpiration have no influence in determining the form of fructification. He denies Klebs' assumption that the hyphae bearing sporangia, and those bearing zygospores, are morphologically equivalent, and shows that the zygospores do not result from the meeting of adjacent outgrowths, but that these outgrowths arise through the stimulation of contact of neighboring hyphae, and are never separate. Falck concludes that with sufficient nutriment the concentration is the special condition effective for zygospore formation. Starting with a normal nutrient solution by the addition of increasing amounts of grape sugar, peptone, glycerine, or mineral salts, a gradual change can be induced from a condition in which sporangia alone are produced, to one in which zygosporic reproduction predominates. Concentration of a normal solution will bring on zygospore production, while if a solution already producing zygospores is sufficiently diluted, sporangia only are formed. When carbohydrates predominate in the nutrient, the harvest depends on the amount of peptone, while the reverse is true when peptone predominates. In the former case the zygospores are black and relatively poor in protein, while in the latter they are brown and the protein per cent is higher.

The publication of the results obtained by Falck led Klebs to test again the effects of relative humidity and transpiration, and in a second paper ('02) he confirms his former conclusion that the appearance of zygospores or of sporangia in different cases depends chiefly on the amount of transpiration, and that the concentration is of secondary importance. He suggests that the opposite results of Falck were due to his working with a different race. That such physiologically different strains of Sporodinia do exist is shown by Falck's (l. c., p. 300) discovery of a form which produced zygospores even in dilute solutions, and would seem therefore to be more like the strain used by Klebs.

It is evident that the conclusions above enumerated are diverse and conflicting to a marked degree. While one author demands excess of moisture or of oxygen as a crucial factor, another postulates conditions exactly the reverse. The seeker for zygospores may choose between an abundant food supply or a substratum poor in nutriment. The presence of parasites is held by one writer to produce, by another to inhibit, zygospore formation. As has already been suggested, the lack of agreement among the results of different investigators is, in the

writer's opinion, due to the fact that their experimentation has been based on the erroneous assumption that external conditions were the controlling factors in the problem, whereas they are in reality either wholly without influence or of quite secondary importance.

In regard to the significance of the phenomena of conjugation it may be said that although from the time of Ehrenberg ('20), the union of gametes has been generally held to represent a primitive sexual process, there have been some who see in this fusion only a vegetative process. Vuillemin ('87) at some length maintains that in his *Zygorhynchus heterogamus* the inequality of the gametes, which he homologizes with sporangia, is due to differences of alimentation and is of no sexual significance. This union he compares to the anastomosis between mycelial cells commonly seen in the clamp connections of the Basidiomycetes, and from the absence in the formation of zygospores of characters which are supposed to be essential to a sexual process he casts doubt on the presence of sexuality in the whole group. Brefeld, though opposing the majority of mycologists in denying sexuality to the Ascomycetes, speaks throughout of the formation of zygospores as a sexual process. Van Tieghem even goes so far as to find an indication of sexual differentiation in the size of the gametes in Rhizopus and Pilaira ('75, p. 81), and in the fact that the forked spines of the zygospores of Phycomyces ('73, p. 295) are produced from one suspensor before they are from the other. Klebs ('98), in his paper on Sporodinia, although recognizing sexuality in the formation of zygospores, does not discuss the subject, and investigates the production of zygospores on the same basis as he would that of any other reproductive form of a polymorphic fungus. Falck ('01, p. 302) merely mentions in a footnote the word "sexuality," and then says that the process of conjugation can be also explained on the basis of mechanical forces, and that the azygospores can as well be sexual.

In regard to the cytology of the zygospore there is little decisive knowledge. The zygote is at first multinucleate, but, according to Léger ('96), with the accumulation of oil in the centre the nuclei entirely disappear, and at opposite poles of the zygospore appear two groups of small granular bodies which are called "Sphères embryogènes." Those in each group fuse together, and the two thick-walled bodies thus formed are the "Sphères embryonnaires" which are to be found in mature zygospores of all the Mucorineae. At germination the "Sphères embryonnaires" increase in size, lose their walls, and fuse with each other. The nuclei which now reappear undergo one mitotic division and

pass into the germ tube. The fusion in the formation of the "Sphères embryonnaires" is looked upon as the only sexual part of the process. On this basis the azygospores are to be considered equally sexual, since they differ from the zygospores only in containing a single "Sphère embryonnaire." These observations of Léger have not been confirmed by Istvanffi ('95) nor by Gruber ('01), who worked carefully on the zygospores of the same form, *Sporodinia grandis*, and Dangeard ('96, p. 255), in collaboration with whom Léger began his investigation, disputes his pupil's later results. The writer also, from an investigation as yet incompleted, of sections of the zygospores of a number of different species, is satisfied that they contain no structures corresponding to "Sphères embryonnaires."

Although the exact nuclear history in the process of conjugation is a matter of great interest, a knowledge of it is not necessary in order to establish the sexuality of the act. Nuclear fusion is considered to be the criterion of a sexual process, yet, as is seen from the nuclear fusion in the ascus and basidiospores, one must have recourse in addition to physiological considerations for an interpretation of the sexuality involved. The results set forth in the present paper show beyond question that sexuality is present in at least a very large percentage of the Mucorineae, and cytological investigation can merely extend our knowledge of the details of a process, the true character of which seems obvious without its aid. It will be of especial interest to determine the cytological conditions in connection with the segregation of the sexes occurring in the formation of zygospores or later, but no systematic experiments with the germination of zygospores have been made during the present investigation. The writer hopes, however, in the near future to be able to present more satisfactory data on the subject.

CITATION OF SPECIES FROM WHICH ZYGOSPORES HAVE BEEN REPORTED.

In the preparation of the ensuing references, recourse has been had to Just's Jahresbericht for the years 1876 to 1902 inclusive, and to the Botanische Centralblatt for 1903 and for the present year up to May 1. For some of the later species Saccardo's Sylloge has been trusted to mention the occurrence of zygospores when they have been reported in the original description, but the compilation of Berlese and de Toni ('88) in Volume VII is of little value in this respect, and fails to mention even the zygospores of *Rhizopus nigricans*. Although the writer has endeav-

ored to accumulate in the following pages all citations which include references to the occurrence of zygospores among the Mucorineae, the literature cited is, no doubt, not wholly complete. It may, however, be assumed to comprise the great majority of citations of this nature.

An attempt to determine in this literature the thallic conditions of the various species enumerated would be manifestly impossible except in connection with those species in which a homothallic condition was made manifest from the figures or descriptions. A figure showing the progametes arising ultimately from the same branch would, if correct, be decisive. Unfortunately those who have done most work on this group of fungi and from whom we should expect the most accuracy have erred in describing and figuring a detail which at the time was not considered of importance. Thus, of known heterothallic forms, de Bary ('66, Figure 2, Plate VII) figures Rhizopus, and van Tieghem ('73, Figure 4, Plate XX) and Bainier ('82, Figure 15) both figure Phycomyces as homothallic; and if Piptocephalis proves to be heterothallic, as is extremely probable from Brefeld's ('72) account of his cultures, this author has figured a similar inaccuracy. That the zygophores can be traced to more or less distant parts of the mycelial growth, though to a certain extent suggestive, is by no means conclusive evidence, since we know that while in homothallic forms the zygophores generally arise comparatively close together, yet in some forms, like Mucors I and II, the zygophores may arise some little distance apart. In the literature appended, an examination of those species for which a heterothallic condition has now been made out will show that it is unsafe to place too great dependence on the accuracy of the figures and descriptions of morphological characters as published.

But aside from the morphological data, the action of the fungus in cultures may become circumstantial evidence of considerable value. If cultures from a single spore or from a single sporangium always fail to produce zygospores, while in cultures from a mass of sporangia zygospores appear, the evidence even without morphological facts points almost certainly to a segregation of sexual characters on separate mycelia. In most of the cases cited, zygospores were found by chance, and no further cultures were made, so that the evidence from the action in cultures is lacking. It has not infrequently happened, however, that zygospores have been found after a sowing on some unsterilized medium which is a common source of the species investigated, and subsequent attempts to continue the growth of zygospores by transfers of spores has resulted in failure. An accidental mixture of sexual strains is the only available explanation of the failure to obtain zygospores in one culture of a given

species when a second culture similar in all respects to the first produces them in abundance. A failure to obtain zygospores by making a gross transfer of sporangia from a zygosporic culture may be due to the fact that one of the strains has short sporangiophores, and thus may escape below the taller growth of the other. To say that all species are heterothallic in which the production of zygospores from spore sowings has not been a constant phenomenon under similar culture conditions, would at present be too sweeping a statement, yet such is undoubtedly the condition in the forms with which the writer is familiar. From actual knowledge and from indirect evidence, the heterothallic condition seems to be the more common among the Mucorineae, and if we are to lay the burden of proof on either side we should be led to consider a species heterothallic until it has been proved otherwise.

Of the 66 forms cited below, the occurrence of zygospores or azygospores in 51 cases has been reported but once, and in the remaining species, a list of which is appended, the number following each form indicates by how many authors the zygospores have been found and reported. — *Mucor Mucedo*, 6; *M. racemosus*, 4; *M. fragilis*, 2; *M. erectus*, 3; *Zygorhynchus Moelleri*, 2; *Phycomyces nitens*, 2; *Spinellus fusiger*, many; *Sporodina grandis*, many; *Rhizopus nigricans*, 5; *Absidia caerulea*, 2; *Pilaira anomala*, 2; *Chaetocladium Brefeldii*, 4; *Piptocephalis Freseniana*, 2; *Syncephalis nodosa*, 3; *Syncephalis Cornu*, 4.

Since the purpose of the present paper is in no sense systematic, no attempt is made to include a special study of the nomenclature and systematic position of the species cited. In so far as possible A. Fischer's ('92) admirable and familiar classification of the group has been followed.

Mucor Mucedo Linné.

"Following a sowing of Helicostylum on dung, zygospores appeared in abundance after twelve days in the recesses where the brownish mycelium was protected against access of air and light." This note of van Tieghem's ('72, p. 1000) is the first reported occurrence of the zygospores of *M. Mucedo*.

Brefeld ('72) states that zygospores never appeared on his slide cultures, though their occurrence was not infrequent in spontaneous cultures of horse dung where he first found them. In speaking of the zygophores he says: "It is fairly certain, however, that they do not arise from two branches of the same hypha as in Sporodinia, since in the youngest condition observed the copulating hyphae could be followed for a considerable distance."

According to Bainier ('83[b], p. 342), if in March and April dried horse dung be examined from a beaten road, it is not rare to find zygospores in the interior, and if laboratory cultures of fresh horse dung arranged in a thin layer be sown with this same *M. Mucedo*, one easily obtains a large quantity of zygospores during this period. Zygospores were obtained many times in January on Bainier's alcoholic decoction of prunes. In his Étude, Bainier ('82) speaks of the zygospores occurring in the dung when the spores of the species are abundant in the horse's food.

Léger ('96, p. 59) obtained the zygospores on horse dung when the substratum had begun to dry: Vuillemin ('82[b]), used zygospores of this form for the study of the zygosporic membrane; and Spegazzini ('91) mentions zygospores in his description of this species from Argentine. The occurrence of zygospores of *M. Mucedo* has not been infrequent on dung cultures in the Harvard Laboratory during the past fifteen years.

The species is heterothallic.

Mucor racemosus Fresenius.

Bainier ('82) found zygospores of this species on moist bread, horse dung, and plaster wet with a solution of glucose, and in a later paper ('83[b], p. 347) he says: "If *Mucor racemosus* be grown in cell cultures in alcoholic decoction of plums during December, January, and February, one infallibly obtains an exaggerated production of zygospores. . . The air was often renewed in the cultures, hence increase or decrease of oxygen can be only an accessory factor." He further remarks that "Since the plant grew on a liquid and in a nearly saturated atmosphere, dryness cannot be a general cause of zygospore production. In solution of peptone or extract of malt zygospores are rare, while in solution of glucose only chlamydospores and sporangia occur." In the plates accompanying this paper (Plate XVII, Figure 6; Plate XVIII, Figure 9) as many as three zygospores are figured arranged in a scalariform fashion one above the other between two adjacent hyphae. No figures showing a homothallic condition are given, and probably such a condition does not exist. Under this species Bainier includes six forms which differ in branching, size of sporangia, and relation to nutrients. One of these gave zygospores only exceptionally. Being of the opinion that polymorphism may exist in this species Bainier considers these as varieties. It is for this reason, perhaps, that the zygospores which he figures as belonging to *M. racemosus* do not correspond and show at least three distinct types (Bainier, '82, Plate I, Figure 11; '83[a], Plate V, Figure

4; '83[b], Plate XVII, Figure 6; Plate XVIII, Figure 9; '84, Plate VIII, Figure 1).

Léger ('96, p. 60) also encountered these zygospores in his researches, but never obtained a sufficient number for study, although he followed Bainier's method during the months said by him to be most favorable for the purpose.

Saito ('04) mentions this species as common on plate cultures exposed to the air. No description is given, but in the three zygospores figured (Plate II, Figure 7) zygophores are represented as belonging to separate hyphae.

Syzygites ampelinus Hildebrand.

Mucor racemosus sec. Fischer.

From a sowing of his *Mucor Vitis* on black bread Hildebrand ('67) obtained zygospores below the sporangial growth. "The origin of the conjugating branches differs from that in Sporodinia, since they never arise in close proximity from the same hypha. They belong without exception to different branch systems."

Fischer ('92) places this form under *M. racemosus*, together with *Mucor Vitis*, with which it is probably connected.

Mucor racemosus, var. brunnea Morini.

According to Morini ('96) the zygospores of this form may be obtained by allowing the nutrient to become gradually exhausted, or by inducing a gradual desiccation of the substratum, though not infrequently zygospores are formed on a substratum rich in nutriment. In all cases the sexual phase supposes full vitality on the part of the fungus, hence the substratum should be conveniently arranged, and in sufficient quantity, so that the mycelium may spread freely. In a suitable temperature, principally from January to April, zygospores form in horse dung, moist bread, and solution of peptone. In horse dung they are most abundant, and form after about ten days. In peptone solution the production of sporangia is abundant, while that of zygospores is scarce. The want of a frequent renewal of air seemed to hinder the development of zygospores, though it favored oidium formation.

Mucor tenuis Bainier.

In this species, described by Bainier ('83[b], p. 353), no zygospores were found, but in decoction of plums during December, January, February, and March, azygospores were produced on erect filaments.

Mucor spinosus van Tieghem.

Bainier ('84, p. 204) obtained the zygospores on alcoholic decoction of pears and plums during the month of August. They are not produced, he says, during the winter. The zygospores are figured (Plate VII, Figure 6), forming in a scalariform fashion between two filaments, one of which bears terminally a columella.

Mucor circinelloides van Tieghem.

Bainier ('84) found zygospores of this species forming in the moist lower portion of a horse dung culture which had probably been sterilized according to Bainier's method with carbon bisulphide. They do not form in decoction of malt nor of prune. The single zygospore figured fails to show the hyphal connections.

Mucor erectus Bainier.

According to Bainier ('84), cultures in prune juice in winter and spring constantly give very numerous zygospores. Double azygospores are as abundant as zygospores. One of the zygospores is figured (Plate VIII, Figure 6), formed from the base of a sporangiophore.

Schröter ('86[b], p. 204) reports that the zygospores of this species were found by Eidam, and Fischer ('92, p. 197) has apparently also observed them.

Mucor fragilis Bainier.

Bainier ('84) states that the zygospores of this species are obtained with greatest ease during the winter and spring in decoction of prunes, and that the culture becomes literally black with them at the end of eight days.

Vuillemin ('04[b]) has used the zygospores of this species in his investigation of the zygosporic membranes.

Mucor mollis Bainier.

Although the zygospores of this species are described and figured by Bainier ('84), their method of formation is not determinable.

Mucor tristis Bainier.
Mucor modestus Bainier.

Bainier ('84) mentions obtaining zygospores of these two species, but descriptions and figures are lacking in both cases.

Mucor neglectus Vuillemin.

In this species Vuillemin ('87) found no zygospores. His figures, however, represent azygospores formed at the ends of recurved branches.

Thamnidium mucoroides Zukal.

This species was found by Zukal ('90) on a moist culture of alligator dung. Not infrequently four to five zygospores are formed in a scalariform fashion between two filaments. The zygospores are formed in the substratum and in the air, but sporangia are not developed from the same hyphae. "The simultaneous occurrence of sporangia and zygospores shows that the alternation of sexual and nonsexual generations has been practically obliterated, and is only indicated by the fact that from zygospores are developed only sporangia, or a sporangium producing mycelium." This is not a Thamnidium, and A. Fischer ('92) rightly places it among the cymose Mucors.

Mucor rubescens Léger.

The zygospores of this species, which was discovered by Léger ('96, p. 69) on beer wort, are said to occur only in the interior of the substratum.

Mucor geophilus Oudemans.

This form of Oudemans and Koning ('01, p. 13) was obtained from a separation culture of forest humus. The zygospores as figured appear to be merely large chlamydospores.

Mucor I Winkler.

This form, experimented on by Winkler ('02), was cultivated from forest earth. "It is a cymo-mucor, very similar to *Mucor alternans*, except that it forms zygospores abundantly on wort gelatine."

Mucor alpinus Hansen.
Mucor neglectus Hansen (not Vuillemin, nor Bainier).

These two species were obtained by Hansen ('02) from soil, the first from the Alps, the second from the Harz Mountains. His *M. neglectus* differs from *M. alpinus* in that the zygospores are produced two days earlier than the sporangia, and the maximum temperature for their development is higher than for that of the sporangia, while the reverse is true in both respects of *M. alpinus*. Numerous cultures were apparently made in investigating the capacity of these forms to produce yeast

cells, and no mention is made of any inconstancy in the formation of zygospores under like culture conditions. Although no morphological description nor figures are given of these forms to which Hansen has given *nomina nuda*, their action in cultures and the fact that they were obtained from the soil which we know is a source of homothallic Mucors, renders a homothallic condition probable.

Mucor flavus Bainier.

This species was found by Bainier ('03, p. 157–9) on decomposing agarics and generally with zygospores. "Upon whatever substance one cultivates this form one easily obtains, towards the end of fall, a large number of zygospores." They were thus obtained on bread, horse dung, agarics, flaxseed flour, and in cell cultures on decoction of horse dung and of prunes. "When the plant decides to give zygospores they are produced in great abundance." They form on sporangial filaments, as in *M. racemosus*, and as many as four may be seen one above the other in scalariform fashion.

Mucor communis Bainier. Mucor prolificus Bainier.
Mucor limpidus Bainier. Mucor vulgarus Bainier.

In these four unfigured species of Bainier ('03) the zygospores are formed in a scalariform fashion on sporangiophores. Zygospores were found during summer in alcoholic prune juice in cell cultures which had been kept in a cool place.

Mucor neglectus Bainier (not Vuillemin, nor Hansen).
Mucor vicinus Bainier.

In these two forms azygospores only formed under the conditions given by Bainier ('03) in the species mentioned above.

Zygorhynchus heterogamus Vuillemin ('03[a]).
MUCOR HETEROGAMUS Vuillemin ('86[a]).

This species was found by Vuillemin ('87) on a spontaneous bread culture. The unequal gametes are cut off from branches of the same erect filament from which also sporangia may develop.

Zygorhynchus Moelleri Vuillemin.

This species was found by A. Möller ('03) from cultures of the mycorrhiza of pine and oak. In 1884 this species came up in the

Harvard Laboratory on a bread culture which had been set for Rhizopus; in 1889 Professor Thaxter in New Haven found the same species growing from a single spore in a van Tieghem cell culture of sterilized peach decoction into which an inoculation had been made from roots infected with peach yellows, and therefore the form was probably of soil origin, and the material which is at present under cultivation was brought by Professor Thaxter from New Haven, where it had been found by Prof. W. C. Sturgis in a separation culture from earth. The writer has examined preparations of all this material, and also of that mentioned by Coker ('03) as occurring spontaneously on a bread culture in the University of North Carolina, and finds them to agree with Vuillemin's ('03[a]) description of *Z. Moelleri*. The species differs from *Z. heterogamus* chiefly in the smaller size of the zygospores, and in the size and shape of the spores. The zygospore formation is essentially the same.

The genus is obviously homothallic.

Circinella umbellata van Tieghem and Le Monnier.

Zygospores were found by Bainier ('03) during a warm period of summer in a culture of fresh liquorice root placed on sphagnum in a cool cellar. They arise on erect filaments upon which sporangia have not been found. Plate VII, Figure 10, of Bainier's paper shows a zygospore the suspensors of which connect with the branches of the same hypha, suggesting the condition in Sporodinia. If the figure is correct in this respect the species belongs to the homothallic group. It will have, however, the distinction of being the only known homothallic form in which the sporangial stage is very common, while the zygospores are extremely rare. The observation needs confirmation. [This is especially true in view of the fact that since writing the above the writer has found that a laboratory culture of this species will form imperfect hybrids with a (+) strain, but is inactive toward a (—) strain. The probability therefore of a heterothallic condition is very great.]

Circinella nigra Bainier.

This species of Bainier ('03) was found by him on horse dung. The zygospores are similar to those of *C. umbellata*, and were found under the same conditions. Plate VII, Figure 9, does not show enough of the hyphae to indicate whether the progametes originate as in *C. umbellata* or not.

Phycomyces nitens Kunze.

Van Tieghem and Le Monnier ('73) were the first to report the zygospores of this species. There is no evidence in the text which would decide the thallic condition of this species, but Plate XX, Figure 4, shows zygophores arising from the same hypha, and it is on the strength of this figure that de Bary ('84, p. 159) classifies Phycomyces with Sporodinia as having zygospores, the progametes of which arise from hyphae organically closely connected, in distinction from Rhizopus and Piptocephalis. Van Tieghem's first cultures of Phycomyces were from cochineal from a certain lacquer factory. These died during his vacation, and the cochineal from the same factory had become sterile, but later he obtained cultures from horse dung, and again from cochineal. In obtaining the zygospores it is probable that a transfer from the horse dung was sown on cochineal which already contained the spores of another conjugative strain, since no mention is made of sterilizing the cochineal. Cell cultures, presumably from a single spore or from a few spores from a single sporangium, never gave zygospores; nor were gross cultures of fruits successful, probably either because they were sterilized, or if not, because they failed to contain spores of an opposing strain.

Bainier ('82) has many times obtained the zygospores upon horse dung mixed with flaxseed flour or soaked with oil. But he adds that often his experiments have not given the expected results. The same author subsequently remarks ('83[b], p. 343), that if during February and March a layer of fresh horse dung 5 to 6 cm. thick in a rather large crystallizing dish is sown with Phycomyces, one obtains the zygospores in abundance after ten to fifteen days or more, according to the richness of the sowing. He has many times repeated this experiment, and has always succeeded during the months indicated. Phycomyces with its zygospores has been distributed by Bainier in Rumeguère, Fungi Gallici 4645; see note in Rev. Myc., x. p. 188.

Although Phycomyces is not uncommon in laboratory cultures, the zygospores have not been reported from this country. Professor Thaxter found them, however, in 1898 in a culture of rabbit dung from Daytona, Florida, and the writer has obtained them synthetically from the two sexual strains (cf. Plate IV).

The species is typically heterothallic.

Phycomyces microsporus van Tieghem.

A single zygospore of this form was found by van Tieghem ('75) on horse dung, and from the sporangium to which it gave rise further cul-

tures were made, but without the subsequent appearance of zygospores. The species, if indeed it is a distinct species, differs from *P. nitens* only in the smaller size throughout. It is possible that this is but the less luxuriant sexual strain to which the germination of the zygospore had given rise.

Phycomyces Pirottianus Morini.

This species was found by Morini ('96) on horse dung in Sicily. The zygospores were rarely obtained, and form only on the surface of the substratum. The progametes are derived from branches which develop each from a separate mycelial hypha.

Spinellus fusiger (Link) van Tieghem.

The brothers Tulasne ('66) were the first to report the zygospores of this species, having found them on *Collybia fusipes*.

Van Tieghem ('75), who also observed them, was able to grow the species only on certain agarics. "The two arched branches which form the zygospores," he says, "come sometimes from the same branch."

Bainier ('82) found the zygospores on the gills of *Collybia fusipes*, and in the laboratory was able to obtain them from cultures on the same host. The zygophores are said to arise sometimes from the same hypha, and the figures distinctly show this condition.

This species appears to be not rare in Europe, and zygospores have been found in connection with the sporangia by all except the earlier investigators of the group. It is not necessary to cite the entire literature on the subject. Writers agree in the constant occurrence of zygospores with the sporangia, and their descriptions and figures point to a homothallic condition. It is of interest to note that the related species *Spinellus macrocarpus*, which in this country has been found by Dr. Farlow on *Mycena Sp.* in Vermont, and by Professor Thaxter on the same host at Kittery Point, Maine, has been kept running for some time in the laboratory, and has never been observed to produce zygospores. The form may well be heterothallic, although the hybridization test has not been applied.

In this country *S. fusiger* has been reported but twice, — Hark. and Moore, Cat. Pac. Fung. 31: 1880, and M. A. Curtis, Bot. N. Car. 153: 1867 (collected by Ravenel). Dr. Farlow has in his herbarium the fungus collected by H. S. Horseford from Charlotte, Vermont, 1886, and collected by himself from the following localities: Shelburne, N. H., September, '97; Campobello, N. B., September, '98; Campo-

bello, N. B., July, '02; Keene Valley, Adirondacks, N. Y., September, '02. Professor Thaxter has the species collected by him from Burbank, E. Tennessee, 1887, and Mr. A. H. Moore collected it on Mount Washington, September, '02.

The writer has examined the material from all the localities mentioned, except that of Harkness and Moore, and is able to substantiate the evidence of the thallic condition shown in the literature. In all the cases the zygospores are abundant between the gills of the host, and the progametes arise at times from branches of the same hypha.

The species is thus homothallic.

Spinellus sphaerosporus van Tieghem.

This form, which has been observed only by van Tieghem ('75, p. 75), is similar to *S. fusiger* in the formation of its zygospores.

Spinellus chalybeus (Dozy and Molkenboer) Vuill.

Vuillemin ('04) separates this species from *S. fusiger*, with which it had been united by Fischer ('92). A tendency toward heterogamy is described, which is indicated by the fact that although the progametes are equal in size, one is of more delicate texture, and after the zygospore is formed, fails to develop a swollen suspensor as does the other. Neither the description nor the figures give us positive evidence as to the thallic condition. None of the five zygospores figured, however, shows the zygophores originating from the same hypha, as is commonly the case in *S. fusiger*.

Spinellus gigasporus Cooke and Massee.

This was found by Cooke and Massee ('89) on decaying agarics from Victoria. The zygospores are borne on slender flexuous zygophoric filaments. The meagre description tells nothing of the thallic condition. Since, however, it is placed in this genus we may assume that the species in question differs little in this respect from *S. fusiger*.

Sporodinia grandis Link.

This is our most common homothallic species, and has been found by all investigators of the group. The descriptions or figures of Ehrenberg (['20] '29), Corda ('39), Bonorden ('51), de Bary ('64), van Tieghem ('75), Bainier ('82), Léger ('96), Klebs ('98), Falck ('01), and others agree in making the progametes commonly originate from branches of the same erect filament. Here, as in Spinellus, the connection of the sus-

pensors is not obscure, and in consequence the figures of any author, even were they not confirmed by so many others, would be more trustworthy than in most cases. From a few experiments on this form van Tieghem ('75) thought that he had established the principle that lack of oxygen is the controlling factor in the production of the zygospores. The papers of Klebs and Falck have already been referred to (p. 218). The species is very common in this country, and cultures have been kept running in the laboratory for several years.

The species is homothallic.

Rhizopus nigricans Ehrenberg.

In de Bary's ('66) paper on *Rhizopus nigricans* is the first mention of the zygospores of a heterothallic form. They were discovered in this species by A. Janowitsch in the Botanical Laboratory at Freiburg. De Bary found zygospore formation occurring during warm weather in May, June, and July, in cultures on fleshy fruits and on bread. On other substrata and in winter only sporangia were observed. In Plate VII, Figure 2, we have the apparently impossible case represented of two conjugating zygophores arising from the same hypha.

Van Tieghem ('75) obtained the zygospores by employing de Bary's suffocation theory. A cylindrical vase, previously washed with boiling water, is filled one-half or two-thirds full with fresh bread crumbs. Upon this are scattered a few drops of boiled water into which *a* sporangium ("un sporange") has been transferred, and the vase is closed. After a dozen days, when the mycelium is sufficiently suffocated by the first vegetation, zygospores appear in the interior of the bread, and especially between the bread and the walls. Our present knowledge of this species indicates that spores from more than a single sporangium must have gained access to the culture, since the form is typically heterothallic.

Eidam ('83) found zygospores of this species in winter on "Erdnusskuchen."

Léger ('96, p. 74) tried van Tieghem's method for obtaining zygospores, but found only a small number in his cultures.

It is of interest to know that de Wevre ('92), in working on this species, attempted to get the zygospores by following the method given by de Bary and van Tieghem, and in addition subjected the fungus to all the unfavorable conditions he could devise, but without obtaining other than sporangial spores. He concludes that either the form with which he worked had lost its sexual capacity or else was a different variety from that which produces zygospores.

The zygospores of this, one of the commonest of our moulds, though infrequently reported, can hardly be rare. They are generally covered by the sporangial growth, and to one unfamiliar with their appearance are distinguishable only with the aid of a microscope. In the fall of 1892 Professor Thaxter found zygospores on a spontaneous bread culture which had been started for the class in Cryptogamic Botany. By transferring from the zygosporic region, the production of zygospores was increased, and the young sporangia appeared unusual in that they showed a distinct orange-yellow color. This fact suggested that this was a special race or strain, and that zygospore formation might be connected with such strains in which for some reason the sexual activity had become apparent. The material was preserved dried on bread from year to year, and could be depended on to produce zygospores when pieces of the old culture were transferred to fresh bread, until the spring of 1901, when the sexual activity appeared to have run out. As the "Harvard strain" this was carried to Barnard College by Prof. H. M. Richards, and to the University of Chicago by Prof. B. M. Davis, and has been used for class work by several laboratories in the country. Professor Richards has told the writer that his scion of the strain ran out at about the same time with the material in the Harvard Laboratory, and the same is true of the material taken to Chicago.

Coker ('03) reports finding zygospores of this species, and has kindly sent the writer material which came originally from a spontaneous bread culture in the University of North Carolina, Chapel Hill, N. Car.

Prof. D. H. Campbell has kindly sent the writer zygospore material which was part of a spontaneous growth on bread exposed in his laboratory at Leland Stanford University, Cal. He writes that he almost always gets zygospores in this way. They were first obtained in 1892 from a spontaneous growth on a piece of squash, and every year since he has had them in abundance.

Zygospores were found in sporangial material from a spontaneous infection of sweet potato kindly sent the writer this January by Prof. F. L. Stevens, of Raleigh, N. Car.

Mr. L. A. Scott, of Cambridge, called the writer's attention to a growth of mould on a mixture of miscellaneous vegetables which had been left together for some days in a jar at the Harvard Botanic Gardens. An examination showed that Rhizopus was the principal fungus, and upon a soft spot in an Irish potato a mass of zygospores was found. This potato was otherwise unaffected, and zygospores could not be found elsewhere among the growth.

After the running out of the original Harvard strain, isolated cases of zygospores were occasionally found in cultures of starchy material in the laboratory, and cultures on paste of the shells of almond nuts have always produced them in abundance.

The species is heterothallic.

Rhizopus necans Massee.

This form is described by Massee ('97) as causing disease of lily bulbs brought from Japan. "Several large spiny zygospores were found in the matted mycelium present in bulbs in the last stage of decay, and presumably belong to our fungus." The single zygospore figured shows no mycelium connections except the halves of the suspensors nearest the zygospore.

Rhizopus Artocarpi Raciborski.

Raciborski ('00) found this species in Java infecting the male inflorescence of breadfruit (*Artocarpus incisa*). The zygospores did not occur on the host plant, but appeared on an agar culture a week old which was contaminated with a Hyphomycete. No figures are given, and it is not impossible that in the laboratory culture where zygospores appeared, their formation was due to an accidental infection with a complementary strain.

Absidia capillata van Tieghem.

The zygospores of this species of van Tieghem were found in the interior of the horse dung and on the lower face of the substratum in contact with the bottom of the plate containing it. The description and figures (Figures 32 and 35) represent zygophores originating from the same branch in close proximity to the future zygospore, and there is no mention of cultures in which zygospores were not found. Such a condition would force us to consider the species homothallic.

Absidia septata van Tieghem.

Cornu found zygospores of this form which van Tieghem ('77, p. 360) refers to *A. septata*, on the lower side of a cork hermetically sealing a flask in which grapevine roots were preserved. "In both cases (*A. capillata* and *A. septata*)," says van Tieghem, "zygospores were produced in an atmosphere poor in oxygen when the vegetation had become sufficiently checked and the formation of sporangiophores completely suppressed by this poverty of oxygen." The description of *A. capillata* as regards the origin of the zygophores is made to apply

to *A. septata*, and Figures 42–45 and 47 would place this species also in the homothallic group. We know with certainty that one species of Absidia (*A. caerulea*) is heterothallic, but this is not a necessary indication that van Tieghem's description of the two species just reviewed is inaccurate, for we have a similar condition in the genus Mucor, where, although the species are predominantly heterothallic, yet homothallic forms are known to exist. It is unfortunate that these two species have not been found by other investigators.

Absidia caerulea Bainier.

Mucor Saccardoi Oudemans.

Proabsidia Saccardoi (Oud.) Vuillemin.

According to Bainier ('89) the zygospores are obtained during the warm season, whenever the substance upon which the fungus develops is laid on a more or less thick layer of a moist absorbent material. Thus "zygospores are always obtained in abundance" when the Absidia is cultivated on bread which is laid on sphagnum, "excelsior," carrot slices, or wood bark.

Oudemans and Koning ('01, p. 13) found this species in February on a separation culture of forest humus made on nutrient gelatine. Zygospores and azygospores are figured. Professor Oudemans has pronounced a specimen of the writer's zygosporic Absidia sent him to be identical with his *Mucor Saccardoi*, and M. Bainier has likewise identified the same fungus with his *Absidia caerulea*. He writes that it is common in the environs of Paris on substances that decay in the woods, and that it is remarkable for the ease with which the zygospores may be obtained. The writer has often found the species on decaying agarics, chestnut burrs, acorns, etc., and together with its zygospores on a culture of rabbit dung. So far as is known it was first found in the United States by Professor Thaxter at New Haven, in 1889, in mixed cultures, and has been several times found by him from various localities in New England, where it appears to be common.

The species is heterothallic.

Pseudo-Absidia vulgaris Bainier.

According to Bainier ('03), "zygospores are sometimes found in horse dung cultures in the lower part of the substratum." The zygospore figured gives no information in regard to the suspensor connections.

Absidia scabra Cocconi.

This form was found on horse dung by Cocconi ('00). When the nutriment in the dung becomes impoverished by the asexual generations, the sexual generation occurs. In the formation of a zygospore "two superficial branches, each of which belongs to a distinct mycelial-hypha, direct themselves toward each other till they meet."

Professor Thaxter has found zygospores of an undescribed species of Absidia on a culture of refuse from a termite's nest from West Africa. In tube cultures made at the time from sporangia, no zygospores developed. In preparations examined of this material the zygophores remain separate as far as they can be followed.

Mycocladus verticillatus Beauverie.

This fungus was found by Beauverie ('00) growing in abundance on a damp wall with many other moulds. The zygospores were found in a culture of the sterile form of *Botrytis cinerea* where the two species developed together with intrication of their filaments. The culture was five months old, and the zygospores were extremely numerous. Beauverie suggests that their abundance may have been because of the support which filaments of the Botrytis furnished. Nothing is said of the character of the substratum except that it had become exhausted by a luxuriant vegetation and had almost completely dried. The single figure of a zygospore has the suspensors incompletely drawn. In the meagre description given us, we have little clue to the thallic condition, except the fact that the zygospores were obtained apparently in but this single culture, although the fungus was subjected to varying conditions in Beauverie's physiological investigations. If this zygosporic culture were the first culture made — a not improbable supposition from its age and admixture with Botrytis — it can easily be seen, upon an assumption of a heterothallic condition, why zygospores should occur here and not later.

Thamnidium elegans Link.

During May and June Bainier ('84) obtained the zygospores of this well-known species in pure cultures in great abundance. They are borne upon erect filaments, which also produce the dichotomies, and a scalariform series is figured (Plate X, Figure 7) between two separate hyphae which are each terminated by a columella.

Dicranophora fulva Schröter.

Schröter ('86') is the only one who has found or described this species which is reported growing on *Paxillus involutus*. The zygospores are formed on the mycelium, which develops abundantly between the lamellae of the host and arise from the copulation of two erect unlike branches. One of them is very thick and sack-like, arising from a mycelial hypha by a narrow stalk, and its upper third is distinguished by a cross wall for the formation of the zygospore. The second branch is hardly thicker than the hypha from which it originates, and from it a short cylindrical piece is cut off. "The unlike character of the copulating branches, especially in size and thickness, points distinctly to a sexual difference in the branches and suggests an antheridium and an oogonium." Figure 113 of Schröter ('97), in Engler and Prantl, leaves no doubt as to the heterogamic character of this species, but the figure is not sufficient to show the connections of the suspensors. The closely related American form subsequently referred to, however, is homothallic.

Choanephora Cunninghamiana Currey.

The species was found by Cunningham ('80) on the flowers of Hibiscus in India. The following statements occur in connection with the description of the formation of zygospores. "This happens comparatively rarely, and I have as yet been unable to determine what the precise conditions are under which it occurs. . . . In all cases in which I have been able to determine the point accurately, the opposed organs have been derived from two distinct mycelial filaments. In many cases, however, the relations of the processes and filaments are very much obscured . . . and in some the appearances seem to indicate that contact occasionally occurred between processes arising from the same filament."

The evidence thus indicates a heterothallic condition.

Choanephora Simsoni Cunningham.

The species was found by Cunningham ('95) on *Ipomoea rubro-caerulea*. In both this and *C. Cunninghamiana* the nutrition was found to regulate the fructification, zygospores being obtained when the nutriment was below the normal. "There is nothing to indicate what is the essential determinant of the appearance of zygospore fructification, for in two cultures side by side from the same specimen of nutrient material zygospores are absent in the one and almost entirely replace the sporangia in the other. . . . In some instances the conjugating processes take origin from

the same mycelial filament, in others from distant ones." Three out of the seven figures of this species are distinctly homothallic in character, and thus oppose the cultural evidence which alone would strongly suggest a heterothallic condition.

Pilaira anomala (Cesati) Schröter.

Van Tieghem ('75) found the zygospores only in two cell cultures, in one of which three spores were sown and in the other five. According to the account given the zygospores form from "two flexuous branches arising from the same lateral branch or from different lateral branches which cross." The zygospores occurred in a cell under favorable conditions for observation, and van Tieghem's description of the origin of the zygophores would make the species homothallic. The fact that in all other cell and gross cultures he never obtained zygospores, added to the fact that where they were found more than one spore was sown, renders the thallic condition a matter of uncertainty.

Brefeld ('81) found zygospores only once, and on horse dung, where the sporangia were luxuriantly developed. Not more than about fifty zygospores were found, and in cultures from the sporangial spores there were no more zygospores produced. The same was true of sporangial sowings from germinated zygospores. The figures are negative as regards the origin of the zygophores with the possible exception of Figure 27, which seems to make the suspensors connect with the same hypha.

Pilobolus crystallinus (Wiggers) Tode.

Zopf ('88) attempted to induce the formation of zygospores by varying the substratum, and used for this purpose both solid and liquid media, but obtained negative results. Sterilized ("ausgekochter") horse dung was placed in a crystallizing dish and inoculated with pure spore material. The Pilobolus at first developed normally, but later was attacked by *Pleotrachelus fulgens* and an undetermined Syncephalis. Zygospores here appeared in abundance, and Zopf concludes that they were caused by the suppression of the formation of sporangia resulting from the attacks of these parasites. In proof of this, the following experiments were made. The spores from sporangia caught pure were sown on sterilized horse dung and gave rise to sporangia alone. Another similar culture was infected with parasites by strewing on it fragments of dung from the original diseased culture. Sporangial formation was checked and zygospores appeared in abundance. It is stated (p. 356): "In Plate

6, Figure 9, the suspensors arise immediately near each other from the same mycelial branch; in other cases they are the ends of longer or shorter hyphae whose insertions lie at a more or less considerable distance from each other and belong to altogether different systems of branching."

Although parasites caused the zygospore formation in this special instance, Zopf thinks it must be possible to cause them by artificial means without the parasites. He expresses his desire to discover the special culture conditions necessary, but as nothing has been published on the subject since, it may be assumed his investigations were fruitless.

Were it not for Figure 9, we should consider the evidence of a heterothallic condition very strong. The other twelve figures of zygospores, however, illustrate rather the condition mentioned in which the progametes belong to different systems. From the presence of Pleotrachelus and Syncephalis in Zopf's zygosporic culture it is obvious that he did not have a pure culture of Pilobolus, and it is conceivable that an opposing strain entered by the same road with the two parasites. His experiments prove nothing, since in the second culture he did not introduce the parasites pure, but on fragments of the very dung that was producing zygospores. If the form is heterothallic he transferred with the parasites the mycelium of the two opposing strains. The account is strongly suggestive of the writer's early work with Mucor III. (p. 286), the zygospores of which first appeared in a tube culture infected with bacteria.

Pilobolus Kleinii van Tieghem.

In one of Zopf's ('92) cultures of *P. Kleinii* on sheep dung the formation of sporangia suddenly ceased, and all the sporangial primordia were found converted into galls infected by the parasitic organism *Pleotrachelus fulgens*. Since in dung cultures of the same Pilobolus where the parasite was absent zygospores were not produced, Zopf concludes that as in *P. crystallinus* the suppression of sporangia by Pleotrachelus is the cause of the zygospore formation. The figures are indecisive as regards the origin of progametes. That the cause assigned by Zopf is not the true explanation is evinced by the fact that Professor Thaxter has found the zygospores of this species on sheep dung but without the parasite. In preparations of this material which the writer has examined, the hyphae in connection with the suspensors remain separate as far as it is possible to follow them.

Pilobolus nanus van Tieghem.

The thick-walled tuberculate "spores durables" which van Tieghem ('76, p. 341, Plate X, Figure 22) describes as developing terminally on short curved stalks from the mycelium, are considered by Fischer ('92, p. 268) to be azygospores.

Mortierella Rostafinskii Brefeld.

According to Brefeld ('81) cultures of this species may be run through a continuous series, perhaps to ten or twelve generations, with exclusively non-sexual reproduction, when zygospores largely take the place of sporangia. In further cultures made with the mycelium or with sporangial spores, he obtained zygospores continuously and in great abundance. There is nothing said which would indicate whether these sporangial transfers were made from a single sporangium or from several, in which latter case sexual strains, if such existed, might have been mixed. From the intricate network of hyphae that surrounds the young zygospore, it was not possible to determine whether the conjugating branches were formed from the same or from two different stolons. The fact that zygospores would not form on slide cultures while they were found on the free walls of his culture dishes is highly suggestive of a heterothallic condition when we remember that it was Brefeld's practice (l. c., p. 11 and 12) in making slide cultures, to use spores from a single sporangium. The zygospores themselves, when placed in a moist atmosphere, did not germinate, but the enveloping hyphae gave rise to sporangia or to new zygospores. The latter condition is not inconsistent with a supposition that the enveloping as well as the conjugating hyphae are from different mycelia.

Mortierella nigrescens van Tieghem.

Van Tieghem ('76) found this species several times in October, 1875, on different agarics, boleti, and on Lycoperdon, and cultivated it on *Agaricus campestris* and truffles. In June, 1876, the fungus was again found and cultivated on *Agaricus campestris* where the zygospores were obtained. "The small tubercle composed at maturity of an envelope of many thicknesses of crowded branches enclosing the zygospore is inserted by a short pedicel upon a filament of the mycelium and sometimes at the point of anastomosis of several different filaments." The morphological observations and figures apparently indicate that the progametes arise in a tuft from a single hypha. It seems strange that if this is a homothallic species, van Tieghem should have been unable to get the zygospores

on *Agaricus campestris* from his several findings of the fungus in the autumn, when he succeeded in obtaining them in the spring on the same substratum.

Chaetocladium Jonesii Fresenius.

According to Brefeld ('81) the zygospores appear in serial cultures after a short period of cultivation. Neither the description nor the figures indicate the hyphal connections of the suspensors.

Chaetocladium Brefeldii van Tieghem and Le Monnier.

Only once from among a large number of spontaneous and artificial cultures which Brefeld ('72) carried on continuously for a long time did the zygospores of this fungus appear, and then on a spontaneous horse dung culture. The Mucor was scant, but the Chaetocladium was very luxuriant, producing side by side conidiophores and yellow zygospores, the genetic connections between which could be easily made out. Brefeld distinctly states that he was unable to discover zygophoric branches arising near each other from a single hypha, although they were found adjacent in great numbers and in continuity with conidiophores. Though attempts were often repeated, zygospores were never obtained from the sowings of spores produced from germinating zygospores.

Pale and dark zygospores of this species were also obtained by Bainier ('84), the color corresponding in either case to temperature differences in the cultures. Nothing is said about the conditions accompanying zygospore formation, and the figures add no information on this point. It is possible, as A. Fischer ('92, p. 287) suggests, that Bainier may have had the zygospores of two species mixed in his cultures.

Schröter ('86[b]) reports that this species is rather frequently found in Breslau with zygospores, and Léger ('96) also mentions their occurrence.

In 1902 the writer found zygospores of this species on horse dung sent from Berlin. They had developed before reaching the laboratory, and it was then impossible to induce further formation. In the spring of 1903 Professor Thaxter obtained zygospores from sowings on sterilized horse dung. The Chaetocladium came from a tube culture, and the Mucor host was taken from a gross culture. This does not render impossible a heterothallic condition, for a complementary strain may have been introduced with the host, and even the tube culture may have contained two strains which were prevented from forming zygospores by unfavorable nutrient conditions.

Syzygites echinocarpus Hildebrand.

This form was found by Hildebrand ('67) on an open moist black bread culture of Arthrobotrys and another mucor. The zygophoric branches arise either both from a lateral branch which dichotomizes near its origin, forming two branchlets which bend toward each other to conjugate: or secondly, the two zygophoric branches arise close together directly from a hypha; or thirdly, they arise from different threads of the mycelium. The first and the third conditions seldom occur. Of twelve figures of zygospores, five show an undoubted homothallic condition. Hildebrand has here and in his *S. ampelinus* made a special study of the origin of the zygophoric branches, and if his account is correct the species is homothallic.

De Bary ('84, p. 163) thinks this may belong to Chaetocladium, and A. Fischer ('92, p. 287) places it under *Chaetocladium Brefeldii*. The figures of these zygospores, however, differ from those of Brefeld, and the thallic condition which is here described has been determined in neither *C. Brefeldii* nor *C. Jonesii*. It would seem unwise, therefore, to connect this form with any known species in the absence of further information concerning it.

Piptocephalis Freseniana de Bary.

The zygospores were discovered by Brefeld ('72) while investigating the parasitic relations of this plant. He does not state whether they were first found in slide or in gross cultures. "It is highly probable that the parasite was incited to sexual reproduction through the especially luxuriant and undisturbed nourishment on the host. The more abundantly it occurred the more in the background did the nonsexual form remain, and this was especially the case if the development received a normal and not too strong impulse through a moderate temperature." Brefeld figures (Plate VI, Figure 19 i) a single spore giving rise to an intricate mycelium which bears both zygospores and conidiophores. The zygophores of one of his zygospores, moreover, can be distinctly traced to the same branch. If this is a correct representation, therefore, the zygospores in *Piptocephalis Freseniana* originate from branches of the same mycelium. Since the importance of this point was not recognized at the time, we can hardly expect in all of Brefeld's elaborate drawings an absolute accuracy as to the finest ramifications, especially in view of the fact that he himself tells us that the condition was much more complex than represented and that in general the mycelial hyphae are

so narrow (1–2μ diam.) that connections can be made out with great difficulty and only by use of stains. In speaking of the zygophoric hyphae he remarks: "In spite of the intricate twisting of the hyphae they can in every case be followed in different directions and belong to different threads of the mycelium." This fact, added to Brefeld's inconstant results with sporangial sowings ('81, p. 75), renders a heterothallic condition probable.

Spegazzini ('91) mentions zygospores in his redescription of this species under the name of *P. arrhiza*.

Pitocephalis Tieghemiana Matruchot.

Matruchot ('99) found this form growing on *Rhizopus nigricans* which had developed upon germinating seeds of pea, bean, etc. This is its only reported occurrence, and neither the description nor the figures give us any evidence as to the thallic condition.

In 1896 Professor Thaxter found a Piptocephalis with zygospores growing in a bread culture from Mammoth Cave, Ky. In the position of suspensor attachments the form resembles *P. Freseniana*, but the zygospores are quite distinct from those figured by Brefeld. Another form found by him in the same year on a Mucor-infected Myxomycete from Kittery Point, Me., has zygospores whose suspensors are attached at considerable distance from each other, and thus resemble those of *P. Tieghemiana*, but the species differs somewhat from Matruchot's description. The writer has examined preparations of these two forms and finds the hyphae connected with the suspensors to remain separate as far as they can be followed.

Syncephalis nodosa van Tieghem.

In Figures 17 and 19, Bainier ('82) shows the conjugative branches arising from the same hypha, and the secondary zygospores are said to be formed from zygophoric branches arising from the same base.

Thaxter ('97) reports the species as being very common in this country, and remarks that one seldom fails to obtain the zygospores in abundance whenever it grows on a copious substratum of other mucors. The figures are negative as regards the origin of the zygophoric hyphae.

Vuillemin ('86^b) used the zygospores of this species in his study of the zygosporic membranes. The writer has several times found this species with zygospores, and it has frequently been found in the laboratory with the same form of reproduction since 1887. He has been unable to con-

firm the statement of Bainier, and so far as the zygosporic hyphae can be traced they remain separate. The difficulties in cultivating this obligate parasite has deferred the determination of its thallic character. The very common occurrence of zygospores would seem perhaps to indicate a homothallic condition.

Syncephalis Cornu van Tieghem and Le Monnier.

Van Tieghem ('75) found the zygospores of this species which had infected a gross mixed culture of *Mucor plasmaticus* and *Pilaira anomala*. "In formation of a zygospore a slender mycelial branch swells at its summit and dichotomizes irregularly much as when it prepares to produce a sporangial tube. While the other branches of this 'palmure' remain short and form expansions like fingers of a glove, two of them grow more in length, place themselves parallel to each other for a very short distance, and generally in contact in their lower part." The text and Figure 88 indicate a homothallic condition. However in Figures 89 to 92, although the zygophores are not traced to their origins, enough is given to show that they arise at least at some distance from the "palmure" mentioned above.

Bainier ('82) describes the zygospores of this species under the name of *S. curvata*. "Zygospores form in the same fashion as in *S. Cornu*, and the description of van Tieghem may apply here." The zygophoric branches are made to arise from the same base or from two distinct filaments of the mycelium, and these two conditions are shown in Figures 9 and 10 respectively.

Léger ('96) reports zygospores, but adds nothing of interest to van Tieghem's account.

Thaxter ('97) describes the mature zygospores which were found on mouse dung, but the origin of the zygophoric hyphae is not shown by the figure.

In February of the present year the writer found abundant zygospores which correspond to figures of this species in a rat dung culture where *S. cordata* and *S. depressa* had also developed their sporangial fructifications. So far as the zygophoric branches were traced they remained separate.

Syncephalis reflexa van Tieghem.

Thaxter ('97) found zygospores of this species on a culture of mouse dung. The figures do not show the origin of the zygophoric hyphae.

Dispira Americana Thaxter.

Professor Thaxter informs me that the zygospore-like bodies found by him in connection with his cultures of Dispira ('95) were probably accidentally associated with it, and that they are doubtless referable to the genus Parasitella recently published by Bainier ('03).

Massartia Javanica Wildeman.

This fungus was found by Wildeman ('97) in the mucus about terrestial algae which had been collected in Java from the bark of a tree and sent him as preserved material. Certain globose double stalked cells are classed as zygospores, and stages of development are mentioned and figures are given, but no other form of reproduction was discovered. These cells, if zygospores, are more suggestive of Piptocephalis or Syncephalis than of any other known forms among the Mucorineae, but the foundation of a new genus on such imperfect data seems undesirable. Although in Saccardo's Sylloge (vol. xiv, p. 437) this is put under the Chytridiaceae, it can best be left among doubtful genera, where Wildeman himself places it. The hyphae connected with the cells in question are separate, as far as shown.

PART II.

In Part I the investigations and theories of previous authors on the subject of the present paper have been summarized, and the results of the writer's own researches have been briefly outlined. It now remains to present in Part II the body of detailed experiments which form the basis of the conclusions already mentioned. It will be convenient to treat the individual forms examined under the thallic groups to which they belong, to which will be added a consideration of the phenomena of hybridization.

HETEROTHALLIC FORMS.

The Mucorineae, as has already been stated in the Introduction, may be divided into two main groups. In the homothallic forms the mycelia are bisexual and the zygospores produced are consequently formed through the interaction of branches of the same mycelium. In the heterothallic group, however, to be considered in this section, the mycelia are unisexual and the zygospores produced are formed therefore through the interaction of branches of two mycelia which are

necessarily different in character. Every species which possesses this type of zygosporic reproduction is capable of being separated into two opposite strains, which may be cultivated separately to an indefinite number of generations without the formation of zygospores, but which will, when allowed to grow in contact on proper nutrients, produce them by means of gametes derived from their respective mycelia. If these strains are sowed side by side, a distinct line of zygospores may be produced when the respective mycelia come in contact. On account of its enormous jet black zygospores thickly beset with forked spines, and the luxurance with which they are produced in a broad line many layers thick, when the two opposite strains of the species are properly contrasted, Phycomyces offers the most striking example of zygospore formation in a heterothallic species.

While in some forms, in addition to the inherent difference between the strains which becomes apparent by the formation of zygospores when they are allowed to come in contact, there is a more or less marked differentiation in vegetative luxuriance, in others the differentiation is less marked. To distinguish the two strains thus differentiated in the heterothallic group, it has been found convenient to use the signs (+) and (−). As has already been mentioned in the Introduction, hybridization will occur only between those strains which have unlike signs, and these designations, it may be mentioned, were first used in distinguishing strains where a vegetative differentiation was apparent. For this reason it has become evident, notwithstanding certain theories to the contrary, that the process of conjugation is sexual in character, and that the (+) and (−) strains represent the two sexes repectively. In several species certain strains have been found which, as far as they have been tested, fail to respond to (+) and (−) strains, the character of which had already been determined in these species, and for this reason they have been called "neutral."

Rhizopus nigricans.

This heterothallic species is the most widely distributed form among the Mucorineae, and shares with Penicillium the doubtful distinction of being the most common fungus weed in laboratory cultures. As has been already mentioned (p. 233), the "Harvard strain," supposed to be a race in which the faculty of zygospore formation was specially developed, has been in use in the principal botanical laboratories in this country, and that the peculiarity of its zygosporic superiority to ordinary Rhizopus has been the subject of some investigation goes without

saying. That heretofore, however, the question has baffled all inquirers is due to the difficulties inherent in the manipulation of this species, and it was not until some time after the real thallic condition was strongly suspected that the writer was able to separate out the two sexual strains.

Preliminary Tests of "Zygosporic Strains."

After the running out of the original Harvard strain the first appearance of more than isolated cases of zygospores in the laboratory was in a paste culture of nut shells which will be called culture "A" to distinguish it from later zygosporic cultures. The zygospores were rather abundant, mixed with other moulds and with bacteria, but by transferring a mass of the zygospores to sterilized bread, and from this again to a third culture, it was possible to obtain eventually a culture containing only Rhizopus. On the supposition that a new zygosporic strain had appeared, pure transfers were made from separate sporangia, but only sporangial growth resulted. As many as thirty pure transfers were made on such nutrients as experience had shown to be productive of zygospores whenever a mass of the zygospores themselves were used for the inoculation, and in only one case — a bread culture — did zygospores appear. It seemed therefore probable that the ordinary Rhizopus, which was not an uncommon weed in the laboratory, had become mixed with the culture, and perhaps from its greater luxuriance had crowded out the zygosporic strain.

To avoid the contamination of these "weed" spores, small masses of the young zygospores with the mycelium adherent were washed and teased out in sterilized water till a microscopic examination showed that they were free from spores, and a few of these zygosporic hyphae were then used in the inoculation of a number of stender dish cultures. In one of these, zygospores were almost exclusively present in the middle of the dish with sporangia only toward the sides, and while the growth was still young the process was repeated, but the cultures were covered with caps of filter paper to insure increased moisture and placed in a chamber kept damp by a bottom layer of wet filter paper. All produced a great abundance of zygospores, which grew up into the paper caps, and before the sporangia had matured, zygospores with attached hyphae were washed, and having been found free from spores were transferred to van Tieghem cell cultures. Although they were finally infected with bacteria and no new zygospores formed, a microscopic examination showed that the few small sporangia which developed came from the hyphae that had been transferred and not from the spores. It seemed thus unquestionable that

the sporangia in these last cultures must be produced from the same hyphae that were connected with the zygospores, yet of five sporangial transfers from these only one gave rise to zygospores, although the conditions of nutrient, temperature, and moisture, were identical. This tube, moreover, furnished no clue to a solution of the problem, since ten pure transfers from it to a variety of favorable substrata failed to produce anything but sporangia. On the original assumption that zygospore formation was characteristic of certain strains this fact seemed to indicate that the sexual character was not distributed throughout the mycelium, but was handed on through the spores of only a part of the sporangia. All attempts, however, to locate these favored sporangia by taking transfers from the lower part of the cultures near the zygospores were as fruitless as were transfers from sporangia higher up. Yet whenever a mass of the sporangia from a zygosporic culture even without the zygospores was transferred, zygospores invariably developed.

TABLE I.

Substratum.	Results.
Egg (yolk)	sporangia; zygospores scattered.
Cocoanut	sporangia; zygospores abundant.
Potato	infected with bacteria.
Apple	sporangia scanty; one zygospore.
Banana	sporangia; no zygospores.
Orange	sporangia; no zygospores.
Horse dung	sporangia; zygospores scattered.
Milk in sponge	sporangia; zygospores abundant.
Urine in sponge	no growth.
Cocoanut milk in sponge	sporangia scanty; zygospores scattered.
Milk in test-tube	sporangial growth 1.5 cm. high; no zygospores.
Urine in test-tube	no growth.
Cocoanut milk in test-tube	sporangial growth 2.5 cm.; zygospores abundant.
Decoction of banana in test-tube	sporangial growth 0.5 cm.; no zygospores.
Decoction of prune in test-tube	sporangial growth 0.5 cm.; no zygospores.

From Petri dish separation cultures which were made to determine whether zygospores could develop from a single spore, separate mycelial "colonies" were transferred to suitable nutrients in tube cultures and in all cases only sporangia resulted, although scattered zygospores occurred in some of the Petri dishes after the various colonies had become indiscriminately mingled. Our present knowledge shows that in those few cases in which transfers from a single sporangium developed zygospores, a contamination of sporangial spores from an opposing strain must in some way have taken place.

During this period of investigation, only a few series of comparative cultures were carried on, since it became evident at the very outset, as was shown by the experiments already mentioned, that the influence of external conditions was a wholly inadequate explanation of the presence or absence of zygospore formation in this species. Such tests as were then made are summarized in the following table. The natural substances mentioned were sterilized in the autoclave at about 125° C., and, after inoculation with a few drops of sterlized water which contained a mixture of spores from a zygosporic culture, were kept covered in a drawer in the laboratory, with the results indicated in Table I.

In the following stender dish cultures shown in Table II, pieces of sponge and masses of sphagnum were cleansed, sterilized, and soaked with the different liquids tested, the inoculations being made from a mixture in sterilized water of spores from a zygosporic culture. It will be seen that the formation of zygospores occurs when such dilute nutrients are used as are available either in a one per cent solution of grape sugar on sponge, or in damp sterilized Sphagnum. On sponge, peptone alone in varying per cents is unable to support the formation of zygospores. Perhaps owing to the dilute solutions used, the sponge cultures did not produce a luxuriant vegetation. In general, however, it is noticeable that the carbohydrate, grape sugar, in contrast with the same per cents of the nitrogen compound, peptone, affords a nutrient comparatively favorable to the production of zygospores.

TABLE II.

Substratum.	Results.
Sponge with distilled water	no growth.
" " dilute prune decoction	sporangia; few zygospores.
Sphagnum with distilled water	sporangia; few zygospores.
" " dilute prune decoction	sporangia; zygospores somewhat abundant.
Sponge with 0.5% grape sugar	few sporangia; no zygospores.
" " 1.0% " "	sporangia; very few zygospores in cavities of sponge.
" " 2.0% " "	sporangia; no zygospores.
" " 5.0% " "	sporangia; few zygospores.
" " 7.0% " "	sporangia few; no zygospores.
" " 10% " "	sporangia; few zygospores.
" " 0.5% Peptone	sporangia scanty; no zygospores.
" " 1.0% "	sporangia scanty; no zygospores.
" " 4.0% "	sporangia scanty; no zygospores.

In Table III nutrients in stender dishes were sterilized as before in the autoclave and inoculated with a mixture in sterilized water of spores

from a zygosporic culture. Those marked *Normal* were placed with their covers on in a locker in the laboratory; those marked *Very Moist* were placed under a bell-jar on a plate lined with wet filter paper, and in addition caps of damp filter paper were fitted over the uncovered stenders; those marked *Dry* had covers removed and were left in the locker in a crystallizing dish loosely covered with a glass plate; and those marked *Very Dry* were uncovered and tightly closed in a glass jar alongside with four stenders filled with calcium chloride. The series showed, more distinctly than is indicated in the table, that increase of moisture increases, and decrease of moisture decreases, the relative abundance of zygospores on a given substratum. On favorable nutrients in very moist air the sporangia were comparatively late in developing,

TABLE III.

CONDITION OF SURROUNDING AIR.

Substratum.	Very Moist.	Normal.	Dry.	Very Dry.
Carrot	sporangia few; zygospores abundant.	sporangia; zygospores.		
Parsnip	white felted growth; sporangia rare; zygospores.	white felted growth; few sporangia; no zygospores.	white felted growth; sporangia dense; no zygospores.	white felted growth; sporangia; no zygospores.
Beet	few sporangia; zygospores.	sporangia; zygospores.		
Sweet potato	few sporangia; zygospores abundant.	sporangia; zygospores.	sporangia; zygospores beneath.	sporangia low and thick; no zygospores.
Banana	sporangia low; zygospores.	scant growth; no sporangia; no zygospores.		
Turnip	sporangia; zygospores.	sporangia; zygospores.	sporangia; zygospores beneath.	
Bread soaked with dilute prune decoction	very few sporangia; zygospores abundant.	sporangia few; zygospores.	sporangia; zygospores beneath.	sporangia; few zygospores in cavities of bread.
Bread soaked with water	few sporangia; zygospores abundant.	sporangia few; zygospores.	sporangia; zygospores beneath.	few sporangia; no zygospores.

and appeared only in the upper parts of the paper cap, leaving below a loose growth of pure zygospores. With a small amount of moisture the zygospores were completely hidden below the well-developed sporangial growth, and in very dry air the formation of zygospores was entirely inhibited, except in the cavities of the bread soaked with prune decoction, where the amount of moisture necessary for their formation had been retained. These conclusions regarding the effect of relative moisture of the surrounding air upon the production of zygospores in Rhizopus have been confirmed by the experience in a large number of cultures both before and since the "sexual strains" referred to later on were separated.

The early study of zygosporic cultures of Rhizopus, some of the experiments in which have been embodied in the above tables, has indicated that the fundamental factor in the production of zygospores is quite independent of the external conditions to which the fungus was subjected. External conditions are seen to have a secondary influence, however. Moisture in the surrounding air is a condition favorable to the formation of zygospores and dryness is unfavorable; while, of the nutrients tested, carbohydrates are more favorable than nitrogenous compounds. In a general way, therefore, the effect of external factors is found to be in agreement with the results obtained by Klebs ('98) with the homothallic form Sporodinia. In the following pages the conditions which are believed by the writer to be of primary importance in the production of zygospores in this species will be considered.

Resolution of Rhizopus into (+) *and* (—) *Strains.*

In June, 1903, an attempt was made to demonstrate the existence of (+) and (—) strains in Rhizopus by opposing a half-dozen pure transfers from a zygosporic culture but without success, while in separation cultures from the same source the zygospores were inconstant in occurrence, and when present were scattered; and on account of the stoloniferous habit of growth of the fungus were prevented from showing even a suggestion of an intermediate line such as has been above referred to as occurring in such forms as *Mucor Mucedo*. On November 20 there were in the laboratory three van Tieghem cell cultures made from zygosporic hyphae, one of which was producing zygospores; three stender dish cultures which had been covered with moist filter paper caps and contained zygospores in abundance; and one gross zygosporic culture on unsterilized bread. From these sources forty-six pure sporangial transfers were made to paste and to potato agar each at such a distance from

the others that zygospores might be expected to appear where some of the mycelial colonies came into contact. Four crystallizing dishes with paste, containing each six transfers, and two stenders with potato agar containing respectively two and three transfers were placed in the warm oven at 26° to 28° C., while three crystallizing dishes with paste containing four transfers each and two stenders with potato agar containing respectively two and three transfers were left at the room temperature. When these cultures matured there was no evidence of zygospores, even though a careful microscopic examination was made of the low-lying hyphae at the areas of contact of all the colonies. On the assumption that Rhizopus belonged to the heterothallic type it seemed strange that out of the forty-six contrasts between pure transfers from zygosporic cultures, in addition to the contrasts previously made, not a single zygospore should have been found. Nevertheless, although the frequent yellow appearance of the young sporangia was suggestive of a varietal difference in the zygosporic strains, the action of the fungus in the cultures above described, as well as the fact that the zygophoric hyphae could never be traced to the same filament, convinced the writer that the species could not be other than heterothallic.

By running a large number of van Tieghem cell cultures into which a small number of zygospores connected with the aerial mycelium had been transferred, it was hoped that the hyphae connected with the two sides of the zygospore would continue their growth and that by dissecting these out under a microscope it might be possible to obtain cultures which one could be sure had the same origin as the two respective gametes, and further that by a contrast on the proper nutrients of the two cultures thus obtained it might be possible to demonstrate the presence or absence of a heterothallic condition. Unfortunately when the hyphae were sufficiently few in number to make out their connections with the suspensors, they failed to grow, and though when larger masses of hyphae were used in the inoculation zygospores were generally developed, it was not possible to trace their mycelial connections.

Finally, in one cell containing a single young zygospore, it was found that the suspensors themselves had germinated, and by transferring the mycelial growths which had thus arisen, two cultures derived from mycelia of different origin, both of which were concerned in the formation of the original zygospore, were obtained. These strains, which for reasons to be mentioned later have been called (+) and (—) respectively, were derived, as we have seen, from the germinations of opposite suspensors. They were inoculated December 1, side by side in a van

Tieghem cell culture of potato agar acidulated with orange-juice, — a nutrient which has the advantage of preventing the growth of bacteria and at the same time favoring the formation of zygospores in this species. Two similar cell cultures were made containing only the (+) and (−) strains respectively, and in these sporangia alone were formed. In the culture, however, in which the (+) and (−) strains were contrasted, from fifty to one hundred zygospores developed, and in several cases the two suspensors could be traced with absolute certainty to the two different (+) and (−) mycelia.

The heterothallic condition of Rhizopus was thus established, and from these two original suspensors a series of (+) and (−) tube cultures have been run to the twenty-seventh generations, and form a certain means of obtaining zygospores at will, whenever spores from the (+) and the (−) strains are sown, either in a mixed condition or side by side on any suitable substratum.

The two strains having been at last obtained, it seemed a matter of some interest to endeavor to discover an experimental explanation of the early failures to obtain zygospores by contrasting sporangial transfers in the paste cultures above described, some twenty-eight of which still remained in the laboratory in five crystallizing dishes. From each dish a mass sporangial transfer was opposed in separate stenders against the (+) and (−) strains respectively obtained in A. The five contrasts of groups of uniform strains against the A(−) strain gave zygospores, while those against the A(+) strain produced only sporangia, thus proving that the twenty-eight transfers were all (+) in character. It is further probable that the same was true in regard to the remaining eighteen of the forty-six original transfers, and that they were (+) in character. The absence of zygospores from the mutual contrasts of these eighteen transfers shows that all in the same culture dish must have been either (+) or (−), and in view of the uniform (+) condition of the twenty-eight transfers tested, it seems unlikely that a (−) condition was present in every case.

By the experiments performed with culture A just discussed, it had been demonstrated that zygospores were produced only through the interaction of the hyphae of the diverse strains A(+) and A(−). Only the two (+) and (−) strains from this one culture were then known, and though such a condition was hardly to be expected, it was perfectly possible that other strains might be found which, on account of having grown under dissimilar conditions, would be sufficiently different in physiological character to form zygospores with both the (+) and (−)

strains which had been determined in the zygosporic culture A. In other words, although the presumption was in favor of considering the (+) and (—) strains sexual in character, it might be found that such was not the case, and that in other zygosporic cultures the opposite strains, if present, were different in nature from those obtained in A. In order to test the prevalence and nature of these strains in the formation of zygospores, a detailed analysis has been made of the following seven cultures, B to H, which were found producing zygospores in widely separated parts of this country. But before further considering these cultures, a brief account of the general procedure used in making them may not be out of place.

It has been found that a temperature of 26° to 28° C. accelerates the growth and favors the production of zygospores in Rhizopus, and therefore in all cases where a different treatment has not been stated the cultures have been run in the warm oven at the temperature above mentioned. Excessive desiccation has been prevented by keeping a dish of water on the same shelf with the cultures. Plain flour paste thickened over a steamer and sterilized in the autoclave has been the nutrient most frequently employed, and small stender dishes, 3.5 cm. in diameter, and 2 cm. tall, have been constantly used, since the zygospores develop in them in greater abundance than in the larger dishes, which are useful for the culture of other forms. The advantage of the smaller dishes may be due to their closely fitting ground-glass covers, which better prevent a loss of moisture. In making contrasts, spore material from the cultures to be tested is inoculated on opposite sides of stenders of flour-paste. Generally within forty-eight hours, when the cultures are run in the warm oven, young zygospores can be readily seen with a hand lens forming a more or less distinct and often yellowish line, clearly indicating the demarcation between (+) and (—) strains wherever they come in contact. In all cases, however, whenever negative results have occurred, the stenders have been re-examined on the fourth day or later.

Culture B was obtained from nut shells from Mrs. J. W. Cushing, Brookline, Mass., and after having been freed from other moulds and bacteria was kept running on bread. A piece of this material which was found to produce abundant zygospores was transferred to nutrient agar December 15, and the day following terminal branches of the mycelium were dissected out in a pure condition from different sides of the culture and transferred to flour paste in stenders. Seven cultures were in this way obtained, and their treatment is indicated in Table IV.

Since when B_7 or B_8 were contrasted with B_2, zygospores resulted, as

indicated in the table, it is apparent that they belong to strains which are opposite to B_2, and by taking into account the fertile as well as the infertile contrasts, it will be possible to arrange the cultures B_1 to B_6 on one side in a column as belonging to the same strain in opposition to B_7 and B_8. By contrasting representatives from the two different strains of this culture B, with the two strains A(+) and A(−), it is seen that the culture under discussion forms its zygospores through the interaction of two strains which have the same value as those previously found in A, and can accordingly be placed under the proper signs in the same column with them.

TABLE IV. Culture B.

Dec. 14. Zygospore mass transferred to orange potato agar.
Dec. 15. Pure mycelial transfers B_1 to B_8 to paste stenders.

Contrasts (Mycelial). Dec. 16.	Results of Contrasts. Dec. 19.	Contrasts (Sporangial). Dec. 18.	Results of Contrasts. Dec. 21.	Strain Equivalents. A (−)	Strain Equivalents. A (+)
$B_1 \times B_4$	no zygs.	$B_2 \times A(+)$	no zygs.	B_7	B_1
$B_1 \times B_8$	zygs.	$B_2 \times A(-)$	zygs.	B_8	B_2
$B_2 \times B_3$	no zygs.	$B_7 \times A(+)$	zygs.		B_3
$B_2 \times B_5$	no zygs.	$B_7 \times A(-)$	no zygs.		B_4
$B_2 \times B_7$	zygs.				B_5
$B_2 \times B_8$	zygs.	$B_2 = A(+)$			B_6
$B_5 \times B_6$	no zygs.	$B_7 = A(-)$			

In the above table, B_1 to B_8 are cultures from transfers made in separating these strains of nut culture B. In the columns marked *Contrasts* is found their disposition, the mark (×) indicating that the cultures between which it is placed were inoculated side by side on flour paste. In the columns marked *Results of Contrasts* is indicated whether zygospores were present or absent as a result of these contrasts. In the columns A(+) and A(−) under *Strain Equivalents* are arranged the cultures from Nut Culture B according to their strain character as determined by (+) and (−) strains of A.

Culture C was obtained on paste from nut shells from Mr. V. B. Swett, Newton, Mass., and for some reason offered greater difficulties to a separation of the strains. As seen from Table V, the two attempts, by using pure transfers from the edge of the mycelium, gave only the single (+) strain. Later, a large number of young zygospores were dissected out and laid separately on nutrient agar in Petri dishes. The following day the suspensors on one side of many of the zygospores, and in three instances those on both sides, had germinated, and made it possible to obtain pure mycelial transfers from them. It should be here noted that in

cases where one of the two suspensors failed to germinate, it was invariably the one belonging to the (—) strain. Including the dissection from the edge of the mycelium, eighteen transfers in this culture were (+) and only three were (—); in culture B, six mycelial transfers were (+) and two (—); while in culture A, probably all of forty-six sporangial transfers were (+) in addition to the transfers from the two opposite suspensors that finally resulted in the separation of the original single (+) and (—) strains. This makes from the three nut cultures a total of

TABLE V. Culture C.

Dec. 8, zygospore mass transferred to potato agar.
Dec. 9, pure mycelial transfers to paste stenders C_1 to C_5.

Contrasts (Sporangial). Dec. 12.	Results. Dec. 15.
$C_1 \times C_5$	no zygs.
$C_1 \times C_2$	no zygs.
$C_1 \times C_3$	no zygs.
$C_3 \times C_4$	no zygs.
$C_3 \times C_4$	very few z.
$C_2 \times C_5$	no zygs.
$C_1 \times A(+)$	no zygs.
$C_1 \times A(-)$	zygs.

C_1 to C_5 = A (+)

Strain Equivalents.	
A(—)	A(+)
C_{16}	C_1
CX_{17}	C_2
CX_{18}	C_3
	C_4
	C_5
	C_6
	C_7
	C_{10}
	C_{12}
	C_{14}
	C_{15}
	CX_{16}
	C_{17}
	C_{18}
	C_{19}
	C_{20}
	C_{21}
	C_{22}

Dec 14, zygospore mass transferred to potato agar.
Dec. 15, pure mycelial transfers to paste stenders C_6 to C_{15}.

Contrasts (Mycelial). Dec. 16.	Results. Dec. 18.
$C_7 \times C_{10}$	no zygs.
$C_7 \times C_{12}$	no zygs.
$C_7 \times C_{15}$	no zygs.
$C_{10} \times C_{14}$	no zygs.
$C_{12} \times C_{15}$	no zygs.
$C_6 \times C_7$	no zygs.
$C_7 \times A(+)$	no zygs.
$C_7 \times A(-)$	zygs.

C_6, C_7, C_{10}, C_{12}, C_{14}, C_{15} = A(+)

transfers C_8, C_9, C_{11}, C_{13} failed to grow.

Dec. 21, young zygospores laid separate on agar in Petri dish.
Dec. 22, pure mycelial transfers made from germinated suspensors into test-tubes C_{16} to C_{22}.

Contrasts (Mycelial). Dec. 23.	Results. Dec. 30.
$C_{16} \times CX_{16}$	zygs.
$C_{16} \times CX_{17}$	no zygs.
$C_{16} \times C_{19}$	zygs.
$C_{16} \times CX_{18}$	no zygs.
$C_{16} \times C_{21}$	zygs.
$CX_{16} \times C_{22}$	no zygs.
$C_{17} \times CX_{17}$	zygs.
$C_{17} \times C_{19}$	no zygs.
$C_{17} \times C_{22}$	no zygs.
$C_{18} \times CX_{18}$	zygs.
$C_{18} \times C_{19}$	no zygs.
$C_{20} \times C_{21}$	no zygs.
$C_{21} \times C_{22}$	no zygs.
$C_{16} \times A(+)$	zygs.
$C_{16} \times A(-)$	no zygs.

C_{16} = A(—)

CX_{16}, CX_{17}, CX_{18} were transfers from the opposite suspensors to C_{16}, C_{17}, C_{18} respectively. Other transfers were from the germinations of single suspensors.

seventy-one (+) and six (−), and of these latter, four came from the suspensors of individual zygospores in cases where both had germinated.

A number of spontaneous infections of Rhizopus in laboratory cultures were tested at this time and found to be (+) in character, a fact which ma le it seem possible that the (−) strain was exotic, and, being carried on the nut shells, either by handling or by infection in the paste cultures, became mingled with the (+) strain apparently predominant in the laboratory. Through the kind assistance of Mr. V. B. Swett it is possible to state that in all probability the two strains are present side by side on the nuts as bought. Several spontaneous cultures of bread and of a number of different vegetables were started by him at the writer's suggestion at the same time with a culture of nut shells on moist bread. The failure of Rhizopus to develop on the other spontaneous cultures indicated the comparative rarity of spores of this fungus in the air of the apartments and rendered probable that its luxuriant growth on the nut bread culture came entirely from spores carried on the nut shells. The zygospores which here appeared were the starting point of *Culture D*, which was separated by taking transfers from two zygospores both suspensors of which had germinated so that two transfers belonged to each strain. The strains obtained produced zygospores when contrasted with the proper strains of *Culture A*.

Culture E was obtained as already mentioned (p. 233) from an infection of a mixture of vegetables at the Botanic Gardens. Pure sporangial transfers were made from a carrot, an onion and a sweet potato, and in addition young zygospores from cultures obtained from this mixed growth were laid on nutrient agar and mycelial transfers E_1 to E_6 were taken from germinations of their suspensors. E_5 was apparently an impure transfer as seen by its giving zygospores with both A(+) and A(−). That zygospores did not show in the tube culture made from

TABLE VI. CULTURE E.

Contrasts. Jan. 9.	Results. Jan. 12.	Contrasts. Jan. 10.	Results. Jan. 12.	Strain Equivalents. A(−)	A(+)
Sweet potato × A(+)	no zygs.	$E_1 \times E_2$	no zygs.	Carrot	Onion
Sweet potato × A(−)	zygs.	$E_1 \times$ A(−)	zygs.		Sweet potato
Onion × A(+)	no zygs.	$E_2 \times E_4$	no zygs.		E_1
" × A(−)	zygs.	$E_3 \times E_4$	no zygs.		E_2
Carrot × A(+)	zygs.	$E_5 \times E_1$	zygs.		E_3
" × A(−)	no zygs.	$E_5 \times E_6$	zygs.		E_4
		$E_5 \times$ A(+)	zygs.		[E_5 mixed]
		$E_5 \times$ E(−)	zygs.		

the original transfer is due to the fact that the potato agar used as nutrient is not a favorable subtratum for their production.

The disposition of these transfers may be seen in Table VI.

TABLE VII. Culture F.

Dec. 22, zygospore material received from Prof. W. C. Coker, Chapel Hill, N. C., and used in inoculating orange potato agar.
Dec. 24, young zygospores laid separate on agar in Petri dish.
Dec. 25, pure mycelial transfers made from germinated suspensors into test-tubes.

Contrasts (Mycelial). Dec. 26.	Results. Dec. 30.	Strain Equivalents. A(−)	A(+)
F I × f_1	zygs.	F I	f_1
F II × f_2	zygs.	f_2	F II
F III × f_3	zygs.	F III	f_3
F II × f_1	no zygs.	F IV	f_6
F IV × f_3	zygs.	F V	f_7
F III × f_2	no zygs.	f_8	F_9
F V × f_7	zygs.		
F V × f_8	no zygs.		
F II × f_9	no zygs.		
f_2 × F_9	zygs.		
F III × A(+)	zygs.		
F III × A(−)	no zygs.		
F V × f_6	zygs.		
F IV × f_6	zygs.		
F II × A(+)	no zygs.		
F II × A(−)	zygs.		

TABLE VIII. Culture G.

Dec. 27, zygospore material received from Prof. D. H. Campbell, Stanford University, Cal., and used in inoculating orange potato agar.
Dec. 29, young zygospores laid separate on agar in Petri dish.
Dec. 30, pure mycelial transfers made from germinated suspensors into test-tubes.

Contrasts (Mycelial). Dec. 31.	Results. Jan. 2.	Strain Equivalents. A(−)	A(+)
G_1 × G_3	zygs.	G_1	g_2
G_1 × G II	no zygs.	G II	G_3
G_1 × G VI	no zygs.	G IV	
g_2 × G II	zygs.	G V	
g_2 × G IV	zygs.	G VI	
G_3 × G V	zygs.	G_7 (germination from a young zygote).	
G II × G IV	no zygs.		
G_7 × g_2	zygs.		
G_7 × G II	no zygs.		
G_7 × A(−)	no zygs.		
G IV × A(+)	zygs.		
G IV × A(−)	no zygs.		

In all the nut cultures, while occasionally one suspensor was larger than the other, the difference was not marked, but in cultures F and G that on one side was regularly swollen to such an extent that its diameter often equalled or exceeded that of the zygospore itself. In Tables VII and VIII a transfer from the germination of a swollen suspensor is represented by a capital letter with the addition of a Roman numeral, as e. g. F II, and a transfer from an unswollen suspensor by a small letter, as, e. g., f_2. Where an Arabic numeral is used with a capital, the suspensors were equal in size. Transfers with the same numerical subscript came from opposite suspensors of the same zygospore. It will be seen that in the two cultures together, eight out of nine of the swollen suspensors taken were from a (—) mycelium and five out of seven of the smaller suspensors belonged to a (+) mycelium. Not enough tests were made to show whether so large a majority of the swollen suspensors are normally (—) as the figures would indicate; but it is significant that since this greater enlargement occurs also on the (+) side the swelling of the suspensor cannot be considered as an indication of sexual differentiation. G_7 was taken from the germination of a young zygote whose walls had just begun to darken, and whose suspensors had shrivelled. The zygospores of Rhizopus, so far as is known, have never been germinated, and in the present instance the word "zygospore" is not certainly appropriate, since whatever ripening process goes on in the zygote may not have been completed in so short a time (two days from inoculation). In culture F, the two strains are equally represented, while in G the (—) strain predominates six to two.

It should be noted that in culture H, Table IX, the (—) strain outnumbered the (+) in the ratio of nineteen to one, while in culture C the (+) strain outnumbered the (—) in the ratio of eighteen to three; and it is further noticeable that the one (+) strain of the former, and the three (—) of the latter were all obtained from germinating suspensors. This fact is of importance since it indicates that in this species the (+) and (—) strains are not distinguished from one another by any difference in the luxuriance of their vegetative development, but that in different cases one or the other may be predominant in that respect.

The (+) and (—) strains in these eight cultures (A to H) having been separated and their character determined by means of contrasts with (+) and (—) strains, chiefly of culture A, it was thought well to confirm the results by using as a standard the strains obtained from the last culture H. Accordingly, April 4, the (+) and (—) strains from each of the seven cultures A to G were contrasted respectively with the (—) and (+)

strains from culture H, and in no case did a growth fail to show zygospores at the line of contact. The constant presence, in all these cultures which have been found spontaneously producing zygospores, of two opposite strains, the individual character of each of which is the same

TABLE IX. Culture H.

(Zygospore material obtained from Prof. F. L. Stevens, Raleigh, N. C.)

Transfers obtained from germinations of single suspensors.

Contrasts. Feb. 12.	Results. Feb. 16.
$H_1 \times H_2$	no zygs.
$H_2 \times H_3$	no zygs.
$H_3 \times H_4$	no zygs.
$H_4 \times H_5$	no zygs.
$H_5 \times H_6$	no zygs.
$H_6 \times H_7$	no zygs.
$H_7 \times H_8$	no zygs.
$H_1 \times A(+)$	zygs.
$H_1 \times A(-)$	no zygs.
$H_7 \times A(+)$	zygs.
$H_7 \times A(-)$	no zygs.
H_1 to $H_8 = A(-)$	

Transfers obtained from sporangia from a culture producing zygospores.

Contrasts. Feb. 19.	Results. Feb. 22.
$H_9 \times H_{10}$	no zygs.
$H_9 \times H_{11}$	no zygs.
$H_9 \times H_{12}$	no zygs.
$H_9 \times H_{13}$	no zygs.
$H_{11} \times H_{13}$	no zygs.
$H_9 \times H_7$	no zygs.
$H_{13} \times H_1$	no zygs.
H_9 to $H_{13} = H_7 = A(-)$	

Transfers H_{18} and H_{19} were obtained from sporangia; H_{15} to H_{17} came from germinations of single suspensors; HX_{17}, HY_{17}, and HZ_{17} were from the suspensors of a single zygospore.

Contrasts. March 12.	Results. March 16.
$H_{15} \times H_{16}$	no zygs.
$H_{16} \times HX_{17}$	zygs.
$HY_{17} \times HX_{17}$	zygs.
$HY_{17} \times HZ_{17}$	no zygs.
$HZ_{17} \times H_{18}$	no zygs.
$H_{18} \times H_{19}$	no zygs.
$HY_{17} \times F(+)$	zygs.
$HY_{17} \times F(-)$	no zygs.
$H_{19} \times F(-)$	no zygs.
$H_{15} \times F(-)$	no zygs.
March 16.	March 20.
$HX_{17} \times F(+)$	no zygs.
$HX_{17} \times F(-)$	zygs.

Strain Equivalents.

A(−) F(−)	A(+) F(+)
H_1	HX_{17}
H_2	
H_3	
H_4	
H_5	
H_6	
H_7	
H_8	
H_9	
H_{10}	
H_{11}	
H_{12}	
H_{13}	
(H_{14} mixed)	
H_{15}	
H_{16}	
HY_{17}	
HZ_{17}	
H_{18}	
H_{19}	

throughout the series, and the coöperation of which is necessary for the process of conjugation, indicates that it is sexual in nature, and that the (+) and (—) strains represent the two opposite sexes.

Investigations of the "Harvard Strain."

A brief history of the "Harvard strain" has been given under the citations in Part I (p. 233), where it was mentioned that the strain, after running for nearly ten years in the laboratories of a number of different institutions, had ceased to produce zygospores. Since, as we now know, this "strain" was merely a chance admixture of (+) and (—) strains which had been preserved in its zygosporic activity by transfers of portions of the substratum containing their spores, it is a matter of interest to discover the effect on the sexual strains of this ten years association, and to learn in what way the culture has lost its power of producing zygospores.

Although since 1901 zygospores have not been obtained in the laboratory from cultures of the "Harvard strain," the material has been preserved on pieces of dry bread. From such an old culture several transfers were made to stenders of flour paste which, by failing to produce zygospores, showed that not more than one of the sexual strains was present. This, by several tests with (+) strains, has been found to be (—) in character, and the same is true of the Harvard material carried to Dartmouth College in 1901 by Prof. G. R. Lyman, which was kindly sent to the writer for examination. In both these instances the zygospores produced by contrasts with (+) strains were perfectly normal.

Dr. Otis W. Caldwell of Charleston, Ill., has kindly sent the writer material which traces descent to the "Harvard strain" through the laboratory of the University of Chicago, and writes that he has been unable to obtain zygospores from it since 1902. A culture from this source fails to produce zygospores when contrasted with (+) strains. With (—) strains, however, although a reaction is evident, accompanied by the formation of numerous progametes, and the characteristic yellowish tinge appears in the zygosporic apparatus, no perfect zygospores are formed. One of the progametes generally remains small, and fails to distinguish its gamete while the other becomes abnormally swollen, often discolored, and more or less tuberculate throughout with a constriction where a septum for the separation of its gamete might be expected. These imperfect conjugations are suggestive of the conditions associated with hybridization, and are similar to what has been observed of one

other culture at least of the Rhizopus material which has been sent to the laboratory.

The fact that in the Chicago culture the strain remaining has (+) characters, while in the culture in the Harvard laboratory the strain remaining has (—) qualities, shows that the running out of these scions of the original Harvard zygosporic culture is probably due to accidental circumstances accompanying the gross transfers of their spores or to contamination from outside strains. The method of perpetuating these zygosporic strains in gross cultures for class work is such that there is no assurance that the culture at the end of the series has any genetic connection with the one at the beginning. Comparatively little can be learned, therefore, from a study of what remains from the "Harvard strain."

Distribution and Thallic Character of Strains in Nature.

The existence of (+) and (—) strains in Rhizopus having been definitely established, it seemed desirable, owing to the ease with which this very common form may be obtained from distant sources, to test its thallic character by a detailed examination of as many and as varied cultures as could be procured, and with this object in view material was collected from various sources and tested in detail, with the results hereafter enumerated.

Associations of (+) and (—) strains of this species have been found producing zygospores in Germany, and France, and have been obtained by the writer from diverse parts of this country, and investigated, with the results set forth in the previous pages. Accordingly questions now arise in regard to the distribution of the individual strains. Requests have been sent to a number of botanists in different parts of this and other countries for spore material from their localities, together with suggestions for obtaining the fungus on spontaneous cultures of bread. Thanks to the kindness of the writer's correspondents, he is able to represent by means of Table X the results obtained by the cultivation of material from a wide range of territory. In cases where more than a single contribution was received from a given locality the material was of different origin, and in the table numerals indicate the order in which the tests were made.

Nearly all the material was received in a dried condition, generally on bread, and inoculations were made into acidulated agar tubes in the writer's lodging where danger of contamination from the laboratory spores was minimized. When the cultures had been freed from the

frequent accompaniment of Penicillium and bacteria, contrasts were made on paste in small stenders which were kept in the warm oven at 26°–28° C. It may be said, however, in this connection that, although infections of Rhizopus are frequent in the laboratory in cultures at all exposed to the air, yet out of between 1500 and 2000 pure tube cultures of different mucors, which the writer has made during the present year in the laboratory where cultures of this species were constantly being opened to the air, only three tubes have shown contaminations by Rhizopus. The chances, therefore, that an admixture of laboratory strains with the material received had possibly occurred may be disregarded in view of the precautions taken.

TABLE X.

Arrangement of Strains according to Sexual Character.

(—)	Neutral.	(+)
Jamaica Plain, Mass. (1) Bread	Brookline, Mass. (2) Bread	Brookline, Mass. (1) Sweet potato
Jamaica Plain, Mass. (2) Bread	Brookline, Mass. (3) Bread	Worcester, Mass. Bread
New Haven, Conn. (2) Bread	Winthrop, Mass. Bread	New Haven, Conn. (1) Baked beans
Lansing, Mich. (1) Bread	Middletown, Conn. Bread	Washington, D. C. Bread
Lansing, Mich. (2) Sweet potato	Granville, Ohio Bread	Delaware, Ohio Bread
Austin, Texas Bread	Breslau, Germany	Madison, Wis. Bread
Nassau, Bahamas Bread	———	
Cambridge, Eng.	Halle, Germany	
———	———	
Berne, Switzerland Bread	Honolulu, H. I. Plant roots	
Nancy, France Quince	Margarita, Venezuela (1) Nuts	
Catania, Sicily Pistachio nuts	Margarita, Venezuela (2) Nuts	
	Caracas, Venezuela Dog dung	
	Port au Prince, Haiti Dung	

Repetitions were made when there was a suspicion that absence of zygospores might be due to bacterial infection or to other conditions unfavorably affecting the growth, and those cultures in which zygospores were not produced under the circumstances mentioned were

placed in the "neutral" column without making any further attempt to obtain positive results by varying the substratum. A number of the cultures in this column showed, before being tested, a certain difference in gross appearance from those in the other columns. This is true of the cultures obtained from the flowers, roots, nuts, and dung, there listed from tropical regions. With these exceptions the majority of the cultures are located in one or other of the sexual columns. Since no critical examination of these forms has been made, the possibility that more than one species is represented among them should not be overlooked. Experiments to determine the actual condition in these neutral strains have not as yet been made.

A few of the (+) and (−) strains constantly form perfect zygospores with the corresponding test strain, but in very small amounts. The strain sent by Professor Cavara from Sicily produces with the (+) strain a distinct line of contact wherein the zygophoric hyphae, young sporangia, and progametes show the characteristic yellow coloration, yet the process of conjugation as in the hybridization contrasts does not go further than the formation of gametes. It is possible that in the wholly neutral strains a similar reduction of the (+) and (−) quality has extended still further to the point of extinction, but it is not certain that their apparent neutrality represents a complete loss of all sexual character. The sexual nature may still remain, and the strains be (+) and (−), although for some reason unable to respond to a sexual stimulus.

The table is not yet sufficiently complete to enable one to determine the relative distribution in nature of the (+) and (−) strains, but enough data are furnished to show that both are widely distributed. It is hoped that a further accumulation of facts will throw more light on the occurrence of these strains in nature.

The following persons have kindly sent, for use in compiling the table, material from the places indicated, and to them the thanks of the writer are due: Miss T. L. Blakeslee, Delaware, Ohio; Prof. O. Brefeld and Dr. R. Falck, Breslau, Germany; Mr. F. I. Brown and Miss L. G. Adams, Brookline, Mass.; Prof. E. A. Burt, Middlebury, Vt.; Mr. G. D. Bussey, Winthrop, Mass.; Prof. O. W. Caldwell, Charleston, Ill.; Prof. D. H. Campbell, Stanford University, Cal.; Prof. F. Cavara, Catania, Sicily; Prof. H. W. Conn, Middletown, Conn.; Prof. F. W. Coker, Chapel Hill, N. C.; Dr. E. B. Copeland, Manila, P. I.; Dr. G. P. Clinton, New Haven, Conn.; Mr. J. W. Curd, Austin, Texas; Prof. J. B. Dandeno, Lansing, Mich.; Prof. Ed. Fischer, Berne, Switzerland; Prof. C. F. Hodge, Worcester, Mass.; Mr. J. R. Johnston and Mr.

L. W. Riddle, Jamaica Plain, Mass.; Prof. Geo. Klebs, Halle, Germany; Prof. C. S. Leavenworth, Shanghai, China; Prof. G. R. Lyman, Hanover, N. H.; Prof. L. Matruchot, Paris, France; Prof. A. C. Moore, Columbia, S. C.; Dr. E. W. Olive, Madison, Wis.; Mr. J. B. Rorer, Washington, D. C., and Nassau, Bahamas; Prof. F. L. Stevens, Raleigh, N. C.; Mr. M. E. Stickney, Granville, Ohio; Prof. P. Vuillemin, Nancy, France; and the writer has himself obtained material from Margarita and Caracas, Venezuela; and Port au Prince, Haiti.

Serial Cultures.

In investigating the (+) and (−) strains of this species a number of serial cultures were conducted to observe the effect of continued cultivation on the sexual activity. December 2, a sporangial series of both strains from culture A was started in tubes of potato agar kept in a culture drawer and the transfers continued up to the thirteenth generations. A similar series was begun at the same time and carried on in the warm oven at 26°–28° C. up to the twenty-seventh generations. From cultures at the room temperature sporangial transfers could be made every third or fourth day, while from those in the warm oven transfers could be made a day earlier. In making transfers a mass of the mature sporangia was generally used, though occasional pure transfers from single sporangia were made in each series. In later tubes from 3 to 4 per cent of grape sugar was added to the nutrient, and increased both the rapidity and luxuriance of the growth. From tubes $A(+)_3$ and $A(-)_3$, the third generations of A(+) and A(−) strains, a mycelial series was started December 11. Each morning a piece of agar with the attached mycelium was transferred with a platinum spatula into a new tube. In this way the growth of the mycelium was uninterruptedly prolonged for over two months and prevented from producing any sporangia whatever until February 19, when the seventy-first tubes were reached and the series discontinued. The last tubes of each of the three series were tested and the growths in them were apparently unaffected in their sexual character or activity, nor had one strain, as in the similar experiment with *Mucor Mucedo* subsequently described, shown any tendency to lose its vitality.

Morphology and Physiology of Conjugation.

In a discussion of the morphology of zygospore formation in this species it will not be out of place to quote from de Bary's ('66) paper on Rhizopus. According to his account zygospores form on low irregularly

branched hyphae which start from the mycelium like stolons whose branches intertwine and develop zygospores at the places of contact and crossing. Here one hypha produces a perpendicular outgrowth which is duplicated by the second at the point of contact with the first. The outgrowths are at first equal in size and have a diameter not greater than that of the hypha which bears them. The gametes cut off are generally unequal in size, one being as long as broad, the other only half as long, and there is generally a marked difference in the size of the mature suspensors, the larger corresponding to the smaller gamete. The zygospores occur entirely separate or in great numbers close together, and are often the only form of reproduction developed from the hyphae that produce them. At times the same hyphae may bear single sporangiophores near the origin of the zygospores.

By growing the two strains opposed in a van Tieghem cell on the proper substratum, it is not difficult to confirm most of these observations of de Bary. In addition to the vigorous stolons which may function as conjugative hyphae, more or less branched slender filaments arise from the mycelia of both strains and may ultimately give rise to sporangia or remain sterile. Where these, however, come in contact with similar growths from the opposite strain, progametes are produced which by their development push apart the fertile filaments. A stolon arising from a node may form progametes if it comes in contact with a filament of the opposite strain, and may later give rise to another node or produce sporangia in close proximity with the zygospores.

When certain (+) and (−) strains are contrasted the young zygospores and the hyphae connected with them are more or less filled with globules of yellow oily material which gives a striking color effect to the line formed by the young zygospores, while between other (+) and (−) strains of this species on the same substratum the young zygospores are colorless. The color when most marked may invade the sporangiophores, and such a culture shows that the young sporangia where zygospores are being formed are bright yellow in color, while those in other parts of the culture are a pearly white at the same stage of development. It has not been determined whether the yellow sporangia are common to both strains, but such a condition is probable, since where the color is present the oily material may occur in the hyphae connected with both suspensors. It will be remembered that the peculiar appearance of these young sporangia on certain nutrients when the "Harvard strain" was first found and when it subsequently reappeared, suggested the idea that zygospore production, in this as well as in other species, might be a

character associated with special varieties or races. The oily material is no doubt a concentrated form of nutriment developed in connection with the storing up of food in the zygospores, but why it should occur only when certain (+) and (—) strains are opposed has not been investigated. The solution doubtless lies in a study of the differences in the individual (+) and (—) strains.

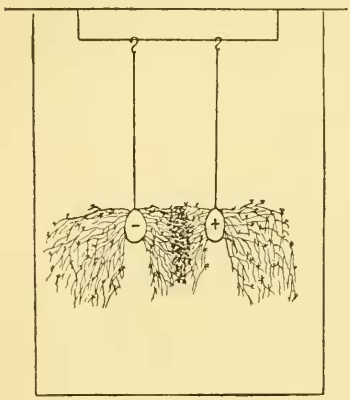
From the spreading habit of this species and from the fact that certain conditions of moisture and nutriment must be satisfied before zygospores will form, it is rather difficult to follow the growth of the zygophoric hyphae. Mutual attraction of these hyphae has been demonstrated in certain species of the genus Mucor; but though the same condition may well hold here, this assumption is not necessary in order to account for the observations which have been made. Whether this be the result of accident or of some attractive force, a contact of hyphae from opposite strains is the immediate stimulus for the formation of progametes, and apparently this contact incites an increased branching in the filaments affected, as is indicated by the fact that zygospores are found in the greatest abundance on intricately branched and interwoven hyphae, especially in the lower parts of the culture where sporangia do not commonly occur.

Moisture in the surrounding air has previously been shown to have a decided influence on the formation of zygospores, and it is probably for this reason that zygospores form preferably between the bottom and sides of the culture dish and the substratum, and especially between layers of filter paper placed over the nutrient for this purpose. The sporangia, on the contrary, are seldom produced in similar places, but rather are formed in the upper parts of the culture. It is not unusual in stender dish cultures to find that the sporangia form chiefly in a thick ring under the edge of the cover, and in test-tube cultures the sporangia are massed in the upper part of the tube while the zygospores are confined to the lower part. In one test-tube culture a crack was found to have occurred at the base of the tube and its location was marked by a distinct line of sporangia which, aside from those in the upper part of the tube, were confined to the limits of the crack. All these facts just mentioned point to something in the outside air which acts as a stimulus to the production of sporangia. Dryness is the factor which suggests itself, and which is considered by Klebs ('98) to be effective in Sporodinia, but test experiments have not been made for Rhizopus.

If the immediate stimulus to the formation of progametes lies in the contact of hyphae from different strains, it probably becomes operative

through the osmotic activity of the hyphal contents. If the surfaces of two membranes in contact become dry there can be no mutual osmotic influence between the fluids contained within them, and it is for this reason, perhaps, that progametes fail to form in a relatively dry air. There are no doubt other causes active, however, an investigation of which the writer hopes to undertake later. The evident advantages of small stenders with close-fitting covers, over large ones with covers that are loose as well as soft over hard agar in connection with zygospore cultures, is probably also due to their influence upon the moisture content of the air.

In order to test the condition necessary for transmission of influence and to determine whether it acts through air or through the solid and moist medium of cultures the following experiment was devised. From the cover of a battery jar, the air in which was rendered moist by a lining of wet filter paper, two bags of cheese cloth containing the substratum (bread soaked in dilute orange juice) were suspended by threads so that they hung free with their inner faces 1¼ inches apart, as is shown in the accompanying diagram. The bags were inoculated with opposite strains, and the apparatus was placed in the dark. In one week zygospores had formed in abundance where the aerial growth radiating from the bags of bread had come in contact. In this experiment any influence upon the origin or direction of growth of the zygophoric hyphae which might have been exerted through the solid culture medium, or which was due to a contact of the masses of vegetative mycelia, was eliminated, and any such influence, if it existed, must have been confined to the aerial branches. If there is an orienting of the zygophoric hyphae, which cannot in any case be a well marked phenomenon in this species, the directive influence must lie outside the substratum and in the hyphae affected.



Differentiation between (+) *and* (−) *Strains.*

In view of the fact that in a majority of the heterothallic forms investigated a morphological differentiation is, as has been previously mentioned, more or less well marked between (+) and (−) strains, it was

hoped that a similar condition might be found in Rhizopus. The unequal swelling of the suspensors has been already referred to as a variable circumstance independent of the (+) or (—) character of the strains contrasted, yet swollen suspensors are almost constantly present in contrasts between certain (+) and (—) strains, while they are as constantly absent in contrasts between others. In addition, conditions of the substratum unfavorable to zygospore formation seem to be a hindrance to suspensor enlargement. Both suspensors may remain small or both may enlarge, and the explanation which naturally suggests itself is that the swollen suspensor is connected with the hypha which happens to contain an excess of protoplasm. Whenever a number of zygospores have been carefully dissected out, the swollen suspensors have always been observed to have the same hyphal connections and are often seen to arise from the larger hypha. In contrast to the opposite filament which, together with the smaller suspensor connected with it, is generally soon empty, the larger suspensor seems to furnish most of the nourishment to the growing zygospore, and it is not unusual to find a young zygospore associated with a suspensor greater than itself and acting as a reservoir of dense protoplasm which it gives up as the zygospore matures. That, however, the difference in size does not correspond to an inherent difference in the (+) and (—) strains as such is shown by its inconstant occurrence as well as by such results as were obtained by culture F (p. 259), in which germinations of both larger and smaller suspensors gave rise to (+) and (—) strains indiscriminately.

Although van Tieghem ('75) was unable to confirm de Bary's view, that there is a relationship between the size of the gametes and that of the suspensors, he considers the inequality in the size of the gametes an indication of a sexual differentiation. The difference in vigor of the two sides is often indicated by an inequality in the progametes; and in cultures where unequal swellings of the suspensors is marked, the difference in their size is generally evident as soon as the gametes are cut off. Though in the material examined the smaller gamete was in a majority of cases adjacent to the enlarged suspensor, the reverse condition has been observed. From Plate I, Figure 15, it will be seen that the size of the gamete can be of no sexual significance in this species. The connected suspensors and their gametes belong obviously to the same strain, and consequently in the two cases where the gametes are unequal the smaller gametes are seen to belong to the opposite strains. This figure is a camera drawing from a preparation taken from a culture where the suspensors were approximately equal in size.

It has now been shown that inequality in the size of the gametes and suspensors is of no sexual significance, and a microscopic examination of both strains shows no appreciable difference in the size and markings of the spores nor in any other of the characters observed. Moreover the gross appearance of their growth in test-tubes and in gross cultures will not enable one to distinguish the strains, and experiments have not been carried on as yet to determine if they possess any secondary physiological characters, in addition to their primary sexual differences.

When the strains of culture A were first separated it was thought that the strain now marked (+) on account of its hybridizing action with other species was the one which showed a greater vigor of growth. In a single bread culture where the (+) and (—) strains were opposed in two parallel lines and zygospores formed at the contact of the opposite growths, it was found that the (+) strain had grown up against the cover, while the (—) strain showed a growth distinctly lower. It seemed possible to explain the exclusively (+) character of all the sporangial transfers taken in separating the strains of culture A by supposing that when sown together there was a constant separation of sporangial growth, the (+) sporangia being in the upper part of the culture where they would be more readily taken for transfers. Further to test this supposition, pure transfers were made from five different sporangia taken from different parts of the upper growth of a bread culture where the spore material used in the inoculation of the two strains had been mixed in approximately equal amounts. All of these transfers, when tested, turned out to be (+) in agreement with the supposition mentioned.

The difficulties which were encountered in obtaining other than the (+) strain in separating cultures A and C (p. 255) pointed to a similar conclusion, as well as the fact that it was definitely shown by a number of tests of spontaneous infections of Rhizopus that (+) spores were the more abundant in the air of the laboratory. Out of nine such infections tested the first five were (+), one was (—), and three belonged apparently to the group of neutral strains previously mentioned (p. 263). Nevertheless, it should be mentioned that in a Petri dish culture which had been accidentally infected from the air of the laboratory it was found that two mycelial colonies had thus originated at opposite edges of the dish and showed that they belonged to different strains by forming zygospores when they came in contact.

In marked contrast to the condition above described, culture H (p. 259), in which the (—) transfers predominated in the ratio of nineteen to one, seemed to indicate here that the relative conditions

were reversed. Accordingly to test the matter further and to determine what if any difference in growth exists between the (+) and (−) strains, on March 23 the (+) and (−) strains of the cultures A to H were mutually contrasted in crystallizing dishes of flour paste, and in addition contrasts were made between (+) and (−) strains obtained from Worcester, Mass., and Nancy, France. All of these contrasts produced zygospores, but although in some the sporangial growth was slightly higher on one side of the dish, this difference had no relation to the places of inoculation, and in none of the cultures could any difference be observed in the growth of the two strains. The only difference, therefore, by which at present the strains of Rhizopus can be distinguished lies in their sexual action when grown in contact.

Mucor Mucedo.

During the past few years the writer has been conducting numerous gross cultures of coprophilous fungi in several of which zygospores have been found which from their general appearance and from the sporangial growth with which they were associated have been referred to that subdivision of the genus Mucor of which *M. Mucedo* may be taken as the type. Although it seems certain that the forms thus associated represent more than a single species, the one which forms the basis of the present discussion appears to agree with Brefeld's ('72) description, and therefore may be assumed to be the typical *M. Mucedo*. It has been generally impossible, when zygospores have been found, to continue their formation in tube cultures by either sporangial or mycelial transfers.

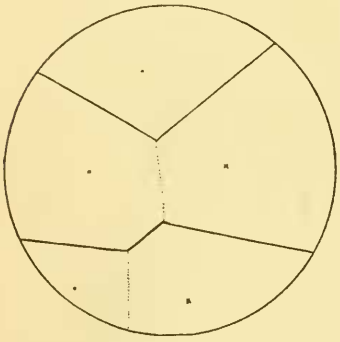
In January, 1903, however, zygospores of this type form were found growing spontaneously on a certain gross dung culture in so comparatively pure a condition that it was possible by making mycelial transfers to hard agar to free the growth from the associated bacteria and to obtain a pure culture of this Mucor with an abundant production of its zygospores. The same difficulty that has already been mentioned of the early cultures of Rhizopus was here experienced in inducing zygospore formation from a sporangial inoculation. If a mass of the mycelium producing zygospores was transferred to nutrient agar, zygospores formed in a dense mass around the point of inoculation, but their numbers soon diminished as the mycelium widened its growth. Transfers from single sporangia never produced zygospores and zygosporic cultures were therefore kept running by mass transfers of the mycelium. Repeated attempts to produce zygospores from mixed sporangial transfers demonstrated the fact, however, that while mixed

sporangial transfers taken from the edge of a zygosporic culture were unsuccessful the reverse was true if similar transfers were taken from the low growth in the centre of such a culture.

Resolution of (+) *and* (—) *Strains.*

In order to discover whether the zygosporic activity could be transmitted through the mycelium arising from any single spore, a separation culture was made of a mixed sporangial transfer from this favorable region in the centre of a culture which was producing zygospores in abundance. This culture was made in three Petri dishes containing a corresponding number of dilutions of the original transfer; and on May 2 (1903) an examination of the dish containing the last dilution in which but five colonies were present, showed the presence of certain remarkable conditions which, by furnishing the first suggestion in regard to what appear to be the fundamental causes affecting zygospore production, have formed a basis for the greater portion of the present investigation.

The conditions referred to are graphically illustrated in the accompanying diagram, in which the crosses indicate the centres of the mycelial colonies which had arisen from five single spores. At the regions of contact between certain of these colonies a luxuriant formation of zygospores was observed, as is indicated by continuous lines in the diagram, while between others in the areas indicated by dots no zygospores were formed. It was soon demonstrated that in every case the branches between which the zygospores were borne could be traced to the individual mycelia on either side of these zygosporic lines just mentioned. The apparent explanation of this unexpected phenomenon was that in this species, or at least in the cultures from which this separation was made, the zygospores are developed through the interaction of individual mycelia which are different in nature.



To determine whether there were more than two distinct strains of these different mycelia, other separations were made from various zygosporic cultures then in the laboratory, and pure cultures up to eighteen in number were secured from the opposite sides of the several

lines of zygospores which resulted. By testing these nine pairs of pure cultures, it was then determined that the mycelium derived from either member of these pairs would take part in the formation of zygospores when grown in contact with the mycelium of a certain member of any other pair. By contrasting all the cultures with one another, it was further found possible to arrange them all in two series in such a manner that those in the opposite series would produce a line of zygospores when grown together, while those in the same series would produce no such line. It may be added that since this time zygospores of this species have been procured from a number of different sources and when the strains producing them have been separated they have always arranged themselves in one or the other of the two series to which, as has already been explained in the Introduction, the terms (+) and (−) have been applied.

The appearence of the zygosporic lines which result when the mycelia of (+) and (−) strains of this species are allowed to grow in contact is illustrated in the culture photographed (Plate IV, Figure 55), where inoculations of the two opposite strains were made as indicated by the (+) and (−) signs. Between the mycelia distinguished by unlike signs a black line marks the position of zygospore formation. The comparatively meagre development of zygospores in the middle of this culture is caused by the less depth of nutrient at this place due to a convexity in the bottom of the Petri dish used for the culture.

The presumption that the formation of zygospores is a truly sexual process, and that in the two strains of this species we have represented the two sexes is substantiated by the results obtained by hybridization to be discussed in a later section. The fact that in this and certain other species the sexes are separated in different thalli led to the use of the term heterothallic to distinguish them from the homothallic forms in which it must be assumed that both sexes are associated in the same thallus.

Morphology and Physiology of Conjugation.

Mucor Mucedo, from the fact that its separate strains have been longest under cultivation, and from the comparative ease with which its growth can be directly observed, has furnished most of the information which has been obtained in connection with a somewhat detailed examination of the process of conjugation. Many points of interest, however, remain untouched, a further examination of which has been deferred for the present.

Cell and slide cultures, which are so advantageous for other forms, are unfavorable to the production of zygospores. In the majority of the observations made, the (+) and (−) strains were contrasted on nutrient agar in Petri dishes, and their growth was examined with the Zeiss A objective. The thin sheets of glass which have been used as covers need to be frequently removed and cleared from the moisture which condenses on their under side from the evaporation of the substratum. It is for this reason perhaps that the best results have been obtained during damp weather, when the drying effect of such an exposure to the air of the room has been lessened.

In the species under consideration, no difference in the growth of the mycelia of the two strains has been observed which can be used to distinguish them. From both arise scattered more or less branched aerial filaments which from the very first are slender and apparently remain sterile, being readily distinguished from the comparatively stout hyphae destined to become sporangiophores, by their more delicate habit, and by the fact that they are not heliotropic. An exaggerated production of these filaments which may occur on certain nutrients often gives a felted appearance to the mycelial growth. Erect filaments of this nature occur throughout the whole mycelial area, but where the mycelia of the opposite strains come together hyphae are produced which, while they are intermediate in size between the sterile filaments just mentioned and the stout young sporangiophores, agree with the former in not being heliotropic.

Direct observation of these hyphae seems to indicate that a mutual attraction, which may be termed *zygotactic*, is exercised between the zygophoric hyphae belonging to opposite mycelia, and they may be seen gradually to approach each other, but in the minority of cases is their contact exactly terminal (Plate II, Figure 30c). In some instances the point of contact may be slightly back of their tips (Figure 32d), or one zygophore may be laterally met by the end of the other (Figures 30b and 33), and in other cases it may even happen that both zygophores touch laterally. At the point where the opposite zygophores come in contact, a swollen progamete is rapidly developed from each hypha, and, according to the position in which the zygophores have met, appears either lateral or terminal. The progametes, however, in all cases are, from the very first, mutually adherent, and by their enlargement push apart the zygophoric hyphae from which they have originated. These latter do not seem to undergo any alteration other than a slight curvature in some instances, occasioned by the lengthening of the progametes.

and further function merely as tubes for the transmission, by way of the suspensors, of nourishment used in the formation of the zygospores.

As has been already mentioned in the Introduction, the account current in text-books states that club-shaped outgrowths (progametes) arise from adjacent sides of hyphae not already in contact and by mutual attraction come to meet at their swollen extremities. In no form which the writer has under cultivation have the gametes been found to be cut off from portions of the zygophoric hyphae which were present before they became approximated. It is only from those structures (progametes), which have developed from the zygophores as a result of their contact, that gametes are delimited. The condition has been correctly interpreted by Falck ('01) from an observation of young stages, and the erroneous opinion just referred to is no doubt due to the difficulty usually associated with any attempt to follow the process by direct observation. Investigators have in general been dependent on a comparison of young stages as a means of reconstructing the process, and have regarded as typical the abnormal conditions frequently encountered in which the apposed progametes have either been torn apart in the dissection of the preparation examined, or, in cases where the zygospores are arranged in a scalariform fashion, have been separated by the more rapid development of a zygospore between the same hyphae.

After the progametes have attained a considerable size there are formed in turn, generally not at the same time, septa which cut off more or less equal terminal cells — the gametes (Figure 29a) — and by the subsequent dissolution of the intervening wall, which proceeds from the centre outwards (Figure 34), an open communication between the contents of the two cells is afforded. The zygote thus formed by an increase in size, a rounding out of its contour, a darkening and denticulation of its outer wall and the formation of a thick layered hyaline one within, assumes the character of the mature zygospore (Figure 35).

In the main the description just given will probably apply equally well to the majority of the heterothallic forms. *M. Mucedo* is peculiar, however, in that its zygophores are sharply differentiated from the sporangiophores and rarely if ever bear sporangia. They are seldom even branched, and a scalariform arrangement of the zygospores, which is characteristic of certain members of the genus Mucor, has never been observed.

The stages in the process of conjugation may be illustrated by the series shown on Plate II, Figures 25 to 32, which consist of camera drawings taken at the intervals noted from a Petri dish culture in the

region where a zygosporic line was commencing to form. In the conjugations (a) and (c) the zygophores met terminally; in (b) a zygophoric branch arose from the (—) mycelium to come in contact with the side of a (+) zygophore which had grown beyond it; and in (d) and (e), the early stages for which are not represented, the ends of the zygophores, when they had grown slightly past each other, were attracted laterally so as to meet somewhat behind their terminations. Two minutes before contact occurred at (d) and while the hyphae were separated by a distance equal to about a third of their width, very slight protrusions were observed on the sides mutually facing (Figure 31), seemingly as if the forces which were drawing the filaments laterally had effected a bulging of the delicate walls at the growing points. Figure 33 is a horizontal view taken from a young zygosporic line, and demonstrates even more strikingly than the vertical view already described, the mutual attraction which the zygophores exert.

The stimulus for the formation of the progametes is thus very evidently the contact between the sexually opposite zygophores, but what is the nature of the stimuli which result in the formation of zygophores and subsequently cause them to approach one another is by no means clear. It has been demonstrated that zygospores would be produced in the case of Rhizopus (p. 270) when only the aerial hyphae of opposite strains were allowed to grow in contact, and therefore that the stimuli to the origin or direction of growth of the conjugative hyphae were communicated solely through the air. From the habit of the species a similar experiment with *M. Mucedo* is much more difficult, and the results which have as yet been obtained are inconclusive.

External Conditions.

No systematic attempt has been made to determine the effects of varying external conditions on the formation of zygospores in this species. In the numerous cultures which have been made, however, it has been observed in general that, as with the other forms investigated, unfavorable conditions check the production of zygospores before that of sporangia. Thus in cell and slide cultures, perhaps because of the thinness of the nutrient layer, and in cultures affected with bacteria, zygospores are not copiously developed. By growing the strains opposed at a temperature of 26°–28° C., it is also possible to prevent conjugation entirely. Moreover, if potato agar, upon which zygospores readily form, be acidulated with orange juice and used as the substratum, no conjugations can be obtained. In general it may be said that in this, as in other cases,

various external conditions have a secondary and variable effect in influencing zygospore formation.

Serial Cultures.

In investigating the persistence of the (+) and (−) characters in the individual strains, the writer has begun a number of serial cultures, and while they are for the most part incomplete and far from satisfactory, the results already obtained are of sufficient interest, it is believed, to warrant a presentation of them even in their present fragmentary condition. In describing the series which were carried on as pure cultures in test-tubes, it will be convenient to represent the series by letters of the alphabet, and the individual cultures in each by the letter of the series followed by the sign of its sexual strain and a numerical subscript indicating the generation which the culture represents.

It has been found that zygospores do not form in the warm oven at 26°–28° C., and in order to test the effect of this unfavorable temperature on the (+) and (−) strains a warm oven series was started February 20, in the tubes $A(+)_1$, and $A(-)_1$. On March 21, the (+) and (−) strains from this series were mutually contrasted. Between $A(+)_2$ and $A(-)_2$, a normal zygosporic line was developed; between $A(+)_3$ and $A(-)_3$, a few zygospores only were found in the centre of their line of contact; while from the contrast between the remaining strains up to A_8, the last generations, no zygospores resulted. It was uncertain from this experiment whether this loss in the power of conjugation, which had been evidently induced by the unfavorable temperature, was equally shared by the two opposite strains, and in order to decide the question contrasts were made from all the (+) and (−) tubes in the series against normally active (−) and (+) strains. Transfers from tubes A $(+)_2$ to $A(+)_5$ were able to take part in the formation of a decreasing number of zygospores, but contrasts against transfers from tubes $A(+)_6$ to $A(+)_8$ produced no zygospores. With transfers made from the (−) tubes it was possible to obtain zygospores only in the case of tube $A(-)_2$. Moreover, when this latter tube was contrasted between $A(+)_3$ and $A(+)_4$, poorly developed white tufted lines occurred at the areas of contact and were found to be made up of arrested stages of conjugation suggestive of the imperfect hybrids discussed in a later section. The line on the side of $A(+)_4$ was slightly better developed and showed its superiority over the other in possessing a single perfect zygospore. A similar contact of $A(+)_2$ between $A(-)_3$ and $A(-)_4$ resulted in neither zygospores nor arrested stages of development and showed, as the contrasts with

the normal strains had also done, that these two later generations of the (—) strain had lost their sexual character before those of the (+) strain. It is apparent that the final disappearance of the (—) characters occurred at or before the time of sporangial formation in tube $A(-)_3$ while the disappearance of the (+) characters occurred at or before their formation in tube $A(+)_6$, or in other words the attenuation of the (—) strain took place three generations before that of the (+) strain under like conditions of cultivation. It remains to be seen whether, along with the disappearance of their power of conjugation the (+) and (—) characters of the two strains have been entirely lost beyond the possibility of being regained by a continuation of the series under more favorable conditions.

The temperature of about 80° F. at which the above series was conducted is not at all unusual during summer months, and an attenuation of a double series of (+) and (—) strains which was carried on during the spring and autumn may be accounted for by the unfavorable effects which the warm weather occurring at that time might have exercised on their growths. That the mere number of sporangial generations, however, through which the strains are conducted has little influence on their sexual power is shown by the unchanged sexual nature in a double sporangial series of the (+) and (—) strains carried on during the winter, the one to the tenth, the other to the eighteenth generations.

In order to discover to what extent the growth of the fungus could be continued by mycelial transfers alone, and to observe the effect of such treatment on the (+) and (—) strains, transfers were made, December 11, into tubes $B(+)_1$ and $B(-)_1$ and formed the starting points for a mycelial series of tube cultures such as was begun on the same date for Rhizopus and has already been described (p. 265). Each morning a piece of the nutrient agar with the mycelium adherent was transferred to a new tube, and the series was continued in this manner, with no perceptible difference in the growths of the two strains, until about the twentieth generation, when, while the (+) mycelium remained normal, the activity of the (—) appeared to be checked, and, on January 5, the mycelium of $B(-)_{25}$ had developed scarcely sufficient growth to enable a transfer to be made, and for several days thereafter underwent but little increase. Eventually, however, this tube produced sporangia, and when tested a month later showed by forming zygospores in contact with a (+) strain that its sexual character had not disappeared.

The growth of the (—) series was renewed by taking a mycelial transfer from tube $B(-)_{23}$ from the upper part of the tube where a mycelium had evidently been developed from fallen sporangia, and for some time

the mycelial transfers grew vigorously. While, however, the (+) tubes remained unaffected the growth in the (—) tubes gradually became again weaker until February 15, when the mycelium in tube $B(-)_{61}$ was almost at a standstill and did not develop sufficiently for a transfer until two days later. The (—) series was then continued on potato agar to which four per cent grape sugar had been added, but the increased nutriment was not a sufficient stimulus to carry the growth well beyond the sixty-fourth generation, when, on February 19, the series was discontinued. The (+) series had then reached the seventy-second generation, and to all appearances had been unchanged either in vegetative or sexual vigor. On the other hand it was ten days before tube $B(-)_{64}$ had developed sufficiently for the formation of sporangia and transfers, made from its spores, in contact with (+) inoculations produced but a feeble mycelium which was unable to take part in the production of zygospores. The same was true of a similar contrast made March 8, but when the experiment was repeated March 13, the growth was better, and a line of zygospores, though somewhat scanty, resulted. From an earlier sporangial culture made from the tube $B(-)_{64}$ zygospores could be obtained by contrast against (+) strains, and it is possible that some of the spores of the March 13th transfer were derived from a mycelium which had developed as a result of a resowing of the tube by the first stunted sporangia formed.

The experiment demonstrates that the continuous cultivation of the mycelium, in so far as conducted, has no apparent influence on the vegetative vigor nor on the sexual capacity of the (+) strain, while it decreases to a marked degree the vegetative vigor, and, as a consequence, the sexual capacity of the (—) strain. The sexual character is not, however, lost, but is merely unable to exert itself when the vital force in the mycelium is thus reduced. The series further offers another example of the general law that unfavorable conditions inhibit the formation of zygospores before that of sporangia.

Germination of Zygospores.

Mucor Mucedo is the only heterothallic species the zygospores of which the writer has succeeded in germinating. In April, 1903, a number of washed zygospores which had been taken from a tube culture of the previous February were sown in a van Tieghem cell. When they were for the last time inspected in the following June, no apparent change had taken place in them, but in October, when the cell was re-examined, it was found that zygospores had germinated and pushed their sporangio-

phores down into the water below, but no growth from any sporangial spores was observed. Transfers from this culture were made into three agar tubes, but in only one of them was there any mycelial growth, and in this single case it was accompanied by bacteria probably derived from the water in the bottom of the van Tieghem cell. By a single sporangial transfer a pure tube culture, however, was obtained from this infected growth, and tests to determine its thallic condition were made during October with five (+) and six (—) cultures, but with negative results. Subsequent tests were made in January of this same tube and of later members of a series of which this tube was the first. All were decidedly (—) in character except the most recent cultures in the series, which appeared to be neutral.

In so far as can be ascertained from the behavior of the cultures in a single experiment of this nature, one may infer that the zygospores from which the series was derived had, in germinating, produced spores, in the mycelium arising from which, the sexual character was dormant, but that the capacity for conjugation became evident after a certain lapse of time. The experiment is unsatisfactory in that the origin of the cultures cannot be traced with absolute certainty to an individual zygospore, although there is not much doubt that they were thus derived. A determination of the time at which the segregation of the sexual characters occurs in strains derived from zygospore germinations, and the nuclear condition associated with it, is a matter of considerable interest. The writer hopes soon to be able to present more satisfactory evidence bearing on the question, and until a greater body of facts is known it will not be prudent to attempt any general discussion of the subject.

Differentiation between the (+) *and* (—) *Strains.*

The (+) and (—) strains of this species offer no morphological differences by which as yet they can be distinguished. In the conjugative apparatus certain differences may exist in the size and time of appearance of the gametes, in the size of the mature suspensors, or in the length of the zygophoric branches, but none of these characters have been found to be correlated with the sexual differentiation of the strains. The shape and size of their spores and their gross appearance in pure cultures are essentially alike in both instances. Although no morphological difference has been determined, yet, as has been shown by the more rapid attenuation of the (—) strain when the two were grown at an unfavorable temperature and by the cessation of growth in the same strain in the mycelial series when the (+) was unaffected, there

is a difference in vigor between the two opposite strains which, as is shown in the section on hybridization, is correlated with their sexual differentiation.

Phycomyces nitens.

The zygospores of this heterothallic species are extremely rare, having been reported, in so far as is known, by but two observers, although the sporangial condition is not uncommon in spontaneous cultures, especially on horse dung. After the secret of zygospore formation was discovered in *M. Mucedo*, the writer endeavored to apply the principle involved to obtaining zygospores of other forms then at his disposal, and contrasts were therefore made between the different cultures of a number of species then growing in the laboratory. By a fortunate chance, the first two cultures contrasted of Phycomyces contained the opposite strains which exist in this species, and consequently a black line of its huge thorny zygospores was produced at the region of contact of the two mycelia.

The zygospores are peculiar, not only on account of the dichotomously branched outgrowths, which arise from either suspensor, but also from the method in which the progametes are developed. Van Tieghem and Bainier, the two investigators who have done the most work on the group here considered and the only ones who have described the zygospores of Phycomyces, have figured stages, showing both suspensors in connection with the same mycelial hypha. Though such an account is incorrect, the condition seemed, *a priori*, not improbable at the time the observations were made, and the fact that the lower portions of the progametes are intricately convoluted and closely adherent renders a determination in every case of their basal connections a task of unusual difficulty, especially when one is dependent on stages obtained from spontaneous cultures. With the two sexual strains in hand, however, it has been comparatively easy to control the production of zygospores and to follow the main steps in the process of their development.

The stages figured in Plate III, Figures 45 to 50, were taken from van Tieghem cell cultures, from the regions of contact of the (+) and (—) strains. In these cultures, no doubt because of the small amount of nutriment afforded in the thin layer of agar used, zygospores have not been formed in abundance, but their development has been none the less easily observed.

When a (+) mycelial hypha is met by one from the (—) mycelium, stout much-lobed branches arise from both at their places of meeting, and become adherent with interlocking of their lobes (Figures 45 to 47), and from the development of two branches thus in contact the pro-

gametes assume their ultimate form. In *M. Mucedo* and in the majority of species in which the process is known, the progametes are in contact only at their extremities. In Phycomyces, however, they grow for a considerable distance more or less intimately adherent with interdigitation of their convoluted lobes, and at this stage form stout yellowish pillars, projecting from the substratum (Figure 48). By the more rapid elongation of the subterminal portion, a separation of the individual components finally occurs below their extremities (Figure 49), but, as is the case in all other forms in which the process has been carefully followed, the ends of the progametes are in contact from the very outset, and never become separate at any stage of their development. The production of the two gametes (Figure 50), and their union to form a zygote, which, by the absorption of nourishment through the suspensors, assumes the form of the mature zygospore, is essentially the same as has been already described in *M. Mucedo*. At about the time of the dissolution of the wall between the uniting cells, outgrowths arise from one or both of the suspensors near their junction with the gametes (Figure 50), and, by their elongation and repeated dichotomy, surround the mature zygospore with a loose envelope of forked spines. Septation in the suspensors or in the afferent hyphae may occur as the zygospore matures, and is also very generally found in arrested stages of development (Figure 49).

The adherence of the basal portions of the progametes is probably due to the fact that the mycelial hyphae are firmly imbedded in the substratum, and their separation by the growth of their gametes is therefore prevented. The presence of a curvature in the progamete of Spinellus when their points of origin are closely adjacent, renders it possible that such a curvature in Phycomyces is also a matter of no great significance and connected merely with the fixed position of the conjugative hyphae. The condition is frequently more simple than is shown in Figure 48, and cases have been observed in which the bases of the suspensors are simply crossed with little or no development of interlocking lobes.

Under the somewhat unfavorable conditions which exist in cell cultures, progametes are not produced at the places of contact between all of the sexually opposite hyphae of the two mycelia, and apparently the quantity of available nutriment has much influence in determining whether a single system of mycelial branches will take part in the formation of more than one zygospore.

The continued growth and branching of the apposed (+) and (−) mycelia would ultimately bring sexually opposite hyphae into contact.

Whether they are also zygotactic, with a mutual attraction influencing their direction of growth, as in *M. Mucedo*, is at present uncertain.

Van Tieghem ('73) was of the opinion that a difference, which is usually apparent in the time of origin of the outgrowths from opposite suspensors, indicates a sexual differentiation. It has been found, however, that this priority in the development of the spines is in some cases on the (+), and in others on the (—) side, and is probably, therefore, associated with a purely accidental inequality in the amount of nutriment received from the afferent hyphae.

Although no differences in structure have been observed, the character of growth of the (+) and (—) strains is distinctly unlike, and it is therefore possible to distinguish them when they are grown side by side. The mycelium in the (—) strain is slightly less vigorous, and the sporangiophores which arise from it are fewer and later in appearance (Plate IV, Figure 52). Moreover, while there is a great variation in the size of the sporangia and sporangiophores derived from the same strain, yet in the (—) the more delicate at first predominate, although in a later period of development no great difference may be discernible between the two strains. On certain nutriments the (—) strain is further distinguishable by an abundant production of sterile filaments from its mycelium, giving to the growth a characteristic white felted appearance.

The effect of external conditions on the activity of conjugation in this species has not been carefully investigated. Zygospores have been secured on all the substrata tested, which fact disproves the generally accepted opinion that oily material is necessary for their production. On nutrients with such low concentrations as are available in potato agar, zygospores are not numerous, but if the concentration is rendered higher by the addition of 4 per cent grape sugar, their formation is considerably increased. They have been also obtained on flour paste, milk agar, and potato agar acidulated with orange juice.

In the warm oven at 26°–28° C., zygospores are produced much less abundantly than at the room temperature.

Neither the apparent inequality in vegetative vigor between the (+) and (—) strains, nor their sexual capacity has been affected by cultivation through twenty sporangial generations.

Since the zygospores of this species were first synthetically obtained, transfers have several times been secured of Phycomyces which has appeared in dung cultures from different sources. Of these strains, two have shown a vigorous (+) character, two a weak (+) character, and one is apparently neutral.

Absidia caerulea Bainier.
Mucor Saccardoi Oudemans.
Proabsidia Saccardoi (Oud.) Vuillemin.

This heterothallic species is decidedly common in occurrence, but has been reported in so far as is known by but two observers. Although Bainier's description is given in Saccardo's Sylloge, Vol. IX this fact seems to have escaped the attention of later writers, and one finds no mention of it, for example, in the recent paper of Vuillemin ('03[b]), in which the genus is considered in some detail and subjected to further subdivision.

As has already been mentioned under the Citations in Part I, the sporangial condition of this species has been frequently found in this country. The first appearance of its zygospores in the laboratory, however, occurred on an old culture of rabbit dung which the writer had collected near Mt. Ktaadn, Me., in the summer of 1902. Less than half a dozen zygospores were found, but by making gross transfers from the region of their formation, their production was increased. Separation cultures, however, made in May, 1903, and contrasts between twelve pure sporangial transfers failed to give the two sexual strains.

In the following fall an attempt was again made to resolve the species into its (+) and (—) strains by obtaining germinations of the hyphae connected with the two suspensors, but the brittle zygosporic branches would not continue their growth when dissected out sufficiently to allow their connections to be followed. By making thirty-eight contrasts between pure sporangial transfers from cultures in which the formation of zygospores was very abundant, four zygosporic lines were finally obtained, and, by transfers from either side of these, the two strains were secured. Later tests have demonstrated that thirty-four of the thirty-eight inoculations contrasted were (—) in character.

The formation of zygospores in this species seems largely independent of external conditions, and zygospores have been obtained on all the substrata tested. In the warm oven at 26°–28° C., the rapidity of growth is increased and zygospores may be secured a day earlier than at the room temperature.

No marked differences have been observed in morphological characters nor in habit of growth between the (+) and (—) strains, although they have been carried in separate tube cultures to the thirteenth generation. It frequently happens that the circinate outgrowths arise from one suspensor before they do from the other, or those on one side may

even entirely fail to develop. Although the matter has not been investigated, it is probable that this peculiarity, as in Phycomyces, has no connection with the sexual differences between the two strains.

Whether there is a mutual attraction between the hyphae from which, at their points of contact, progametes are developed, is at present uncertain. That such an assumption is not necessary, however, to account for the facts observed, is shown by the results of an experiment similar to that already described under Rhizopus (p. 268). In this instance bags of nutriment were freely suspended so that their opposite faces were 3 cm. apart and were inoculated respectively with (+) and (−) strains of the Absidia. Zygospores formed in abundance where the hyphae from the opposite strains met in the air space between the suspended masses of nutriment and thereby showed that, if any orienting of the conjugative hyphae occurs, the directive influence must lie outside the substratum and in the hyphae immediately affected.

Sufficient evidence is not at present available to determine the relative abundance in nature of the (+), (−), and neutral strains. Of three additional strains of this species derived from different sources, however, one is weakly (−) in character, and two are apparently neutral.

Mucor n.

This heterothallic species, which is the type of a new genus, was found by Professor Thaxter together with its zygospores on a laboratory culture and kindly given the writer for experimentation.

External factors have more influence on zygospore formation in this species than in most of the other heterothallic forms under cultivation. At the room temperature under rather dry conditions, a zygosporic line is slow and feeble in development. If, however, the cultures are conducted in a nearly saturated atmosphere, zygospores are more abundant, while, in the warm oven at 26°–28° C., the line is decidedly thicker and earlier in appearance. Concentrated nutrients, in so far as they have been tried, fail to yield zygospores.

When contrasted, the (+) and (−) strains show a striking difference in habit which readily enables one to distinguish them. The growth of the (−) strain is lower and almost white, while that of the (+) strain is higher and dirty yellow in color. These differences between the (+) and (−) strains as well as their sexual character have remained unchanged through sixteen sporangial generations.

Mucor III.

The production of the zygospores of this undescribed Mucor, which apparently belongs to the *M. racemosus* group, has been continued under cultivation probably longer than those of any other heterothallic form, with the exception of the "Harvard strain" of Rhizopus.

The zygospores of this species were first found in a test-tube culture infected by bacteria, which had been made from an impure transfer from rat dung in an attempt to obtain an inconspicuous Mucor which was concealed below the growth of what appeared to be a larger form. Since it seemed not impossible that the production of zygospores might in some way be connected with a change in the character of the substratum induced by the accompanying growth of bacteria, tests were made by starting cultures from sporangial transfers to which bacteria were added. The results of these cultures were, however, negative, since no zygospores appeared. The original mixed culture was eventually freed from bacteria by successive mycelial transfers to solid media, and continued to produce zygospores whenever masses of mycelia were transferred from the zygosporic region.

Similar experiments as to external conditions were carried on for Mucor III, which have been already described for Rhizopus, but the zygospores were more uncertain in their occurrence. In general, nutrients with higher concentrations seem to support a greater abundance of zygospores than those with lower concentrations. Few substances have been tested, but flour paste, plain bread and bread soaked with dilute prune decoction, potato agar and potato agar plus 4 per cent grape sugar, have all been successfully used as substrata for the production of zygospores. On plain agar, horse dung agar, and potato agar acidulated with orange juice, however, it has not been possible to obtain zygospores.

When the cultures are grown in the warm oven at 26°–28° C., the formation of zygospores appears to be more abundant than when the same substratum is used at the temperature of the laboratory.

This species was finally resolved into its two sexual strains by making separation cultures of zygosporic material which had been preserved in a dried condition on bread. Five paired colonies were obtained separated by zygosporic lines, and in all cases there was a more or less marked difference apparent in habit of growth between members of each pair. Later tests have shown that, in every instance, the cultures with the lower development of sporangia have belonged to the strain which has

been called (—). The separate (+) and (—) strains have now been carried to fourteen sporangial generations, and neither the vegetative difference between them nor their individual sexual character has shown any alteration.

Plate IV, Figure 58, is a photograph from an agar culture in which the two opposed strains were in the process of forming zygospores at their line of contact. The growth of the (+) strain on the left is much taller, looser, and lighter in general appearance than the other, while that of the (—) on the right is low and dark. Although, when the two strains are grown side by side, they are always distinguishable by their vegetative appearance, the difference is not equally marked under all conditions. A microscopic examination shows that this diversity in aspect between cultures of the two strains is correlated with a corresponding and striking difference in size of the vegetative reproductive bodies, the smaller spores being formed by the (—) strain. Plate I, Figures 23 and 24, are camera drawings representing the normal variations in size and shape of the spores taken from (+) and (—) potato agar tubes which had been kept under the same conditions of cultivation. It is here seen that the spores produced by the low growth of the (—) strain are smaller than those produced by the more luxuriant growth of the (+) strain. The difference in vegetative luxuriance between the (+) and (—) strains of Mucor III is greater than between those of any other form whose strains have yet been separated, and coupled with the difference in spore characters would almost certainly have been considered by systematists as of specific value.

Mucor IV.

The zygospores of this heterothallic species were found in the fall of 1902 in an old tube culture of potato agar into which the previous summer at Cold Spring Harbor, L. I., the writer had made a transfer of one of the Cephalideae together with a weft of Mucor hyphae to furnish a host for its development.

Separation cultures on potato agar, in the attempt to resolve this species into its sexual strains, failed to produce zygospores in any of the dilutions. The (+) and (—) strains were finally separated, however, by making thirteen contrasts on horse dung agar, from pure sporangial transfers taken from a gross dung culture in which zygospores were abundant. Inoculations taken from either side of the single scanty line of zygospores that resulted formed the starting points for a sporangial series which has already been carried to the fourteenth generations

without having suffered any apparent change in their sexual character. No essential differences have been observed between the (+) and (−) strains aside from that shown in their capacity for conjugation.

At the temperature of the laboratory, zygospores do not form on the artificial substrata usually employed as culture media. In the warm oven, however, at a temperature of 26°–28° C., zygospores can be obtained on potato agar the concentration of which has been increased by the addition of 4 per cent grape sugar, but on the same substratum at the room temperature zygospores are not produced. In so far as experiments have been tried, therefore, the optimum of temperature and of concentration is higher for Mucor IV than for the other heterothallic forms under cultivation.

Mucor V.

Zygospores of this heterothallic species were first found in a small patch on a spontaneous dung culture in the laboratory, and by separation cultures and pure sporangial transfers it was eventually resolved, March 7, into its (+) and (−) strains. Mucor V seems to be sexually the most vigorous heterothallic form that the writer has under cultivation, and no substrata have been tried on which it will not form its zygospores in abundance when growths from the (+) and (−) strains are apposed.

In prepairing the culture photographed (Plate IV, Figure 53), inoculations of the two opposite strains were made at the places indicated by the (+) and (−) signs. The zygophoric hyphae seem strongly zygotactic, and, recurving downward where the (+) and (−) growths come in contact, form a furrowed line of crowded zygospores. The zygophores are of a more or less indefinite growth, and the zygosporic region is soon extended on either side of the original areas of contact (Figures 53 and 57) in curved lines of light brown zygospores which ultimately cover the whole surface of the culture as the two strains become completely intermingled. White lines are observable in the photographs at the region of contact between the opposite strains. They are always present shortly after the first zygospores have been formed, and consist of zygospores in arrested stages of development.

A detailed investigation of the process of conjugation in this species has not been attempted, but essentially the same condition prevails as has been already described in *Mucor Mucedo*. Plate III, Figures 40–44, are camera drawings taken at the intervals noted from a van Tieghem cell culture which had been inoculated with a mixture of

spores from the (+) and (−) strains. The zygophores which gave rise to the progametes shown in Figure 40 a were observed, about an hour before the drawings were commenced, to be in the same relative position to each other as those figured in Figure 40 b. In both conjugations (a) and (b) the zygophoric hyphae met nearly terminally, while the free hypha at (c) grew into contact with its complementary zygophore from which three progametes had already been developed. In Mucor v, a continuation of the growth of the zygophores after the first contact is apparently the rule, and a consequent scalariform arrangement of the zygospores very frequently occurs (Figure 44 b).

The most noticeable vegetative difference between the (+) and (−) strains lies in the time of appearance of the sporangia. When the culture photographed (Plate IV, Figure 53) was two days old, sporangia had appeared in the growths from all the (+) inoculations, but even an examination under the low power of the microscope failed to show any from the (−) growths, and it was not until the day following that they were observed.

Mucor vi.

The zygospores of this heterothallic species were first observed in February last in a separation culture of a Mucor from a laboratory dung culture, and the zygospores have been subsequently found on a later spontaneous culture.

Except in point of size, the individual zygospores resemble those of *M. Mucedo*. The zygophoric hyphae are commonly branched, longer, and less distinguishable from the sporangiophores. They are strongly zygotactic, and the zygophores which developed in the region of contact between the (+) strains in the culture photographed (Plate IV, Figure 54) showed from the centre of these neutral lines where the filaments were upright, an increasing curvature toward the (−) growth, separated from them by the lines of young zygospores.

The photograph fails to show the greater height of the (+) growth, which is often distinctly noticeable under certain conditions of cultivation, but the difference between the two strains is never so marked as in the case of Mucor iii.

Summary of Heterothallic Forms.

In the above nine species, which represent five distinct genera of the Mucorineae, it has been shown that the formation of zygospores in all cases results only through the interaction of two differing unisexual mycelia which have been termed (+) and (−).

In the majority of the forms, the (+) strain is characterized, in comparison with the (—), by a greater vegetative luxuriance. In Mucor III the difference is most distinct, being markedly shown in the size of the spores and in the height of the sporangial growth; in Mucor N the difference is in color as well as in the height of the sporangiophores; in Mucor V, the sporangia from the (—) strain are produced later; in *M. Mucedo*, the difference is discernible only through cultivation under unfavorable conditions; while in some others, as Rhizopus, no differences in vegetative characters have yet been observed.

In *Mucor Mucedo* the sexual strains have been rendered neutral in their action by cultivation under unfavorable conditions, and in Phycomyces, Absidia, and Rhizopus, strains have been found which from the first have shown themselves neutral in character.

External conditions have only a secondary influence on the formation of zygospores and affect the various species differently. Thus, for example, while a temperature of 26°–28° C. favors zygospore production in Mucor III and Mucor N, it entirely prevents their production in *Mucor Mucedo*, and, although a comparatively high concentration seems to be necessary for the formation of the zygospores of Mucor IV, it is detrimental to the production of those of Mucor N.

In all the forms the stimulus for the development of the progametes is the contact between sexually opposite hyphae. In *Mucor Mucedo* these arise from the mycelium as differentiated zygophores, and seldom or never bear sporangia; in Absidia and Rhizopus there is no evident differentiation into zygophoric hyphae, and apparently any of the undifferentiated aerial hyphae may take part in the formation of zygospores; and finally in Phycomyces the progametes arise from the contact of branches of the vegetative mycelium. In *M. Mucedo* and others of the genus Mucor, a mutual attraction between the zygophores is demonstrable, but in some forms, as Rhizopus, it has not been determined.

HOMOTHALLIC FORMS.

As has been stated in the Introduction, those species have been called homothallic the zygospores of which originate from a single mycelium in distinction to the heterothallic species, the zygospores of which are formed by a union of gametes which have originated necessarily from two different mycelia. It has been currently assumed that all species belong to the first class, and consequently the distinction above given has not been hitherto recognized. An enumeration, however, of those

forms in which the thallic condition is known or strongly suspected will show that the homothallic group undoubtedly comprises a very small minority of the species in which zygospores have ever been found. These forms may be recognized by the fact that they can be induced to develop zygospores constantly on the same substrata when grown from a single spore, and, moreover, by the fact that through the zygophoric hyphae the opposed progametes may be traced to the same branches of the mycelium.

The members of the homothallic group may be conveniently divided into two subsections according to the presence or absence of a constant morphological difference in the gametes and zygophoric branches. The first division comprises Sporodinia, Spinellus, and Mucors I and II, and the second comprises Zygorhynchus and Dicranophora, both of which have been classed together as heterogamic on account of the morphological differentiation in their gametes. The homothallic forms will be considered in the order just given.

Sporodinia grandis.

Inasmuch as *Sporodinia grandis* is by far the most abundant and widely distributed homothallic form among the Mucorineae, and therefore the only one from which earlier workers could be sure of obtaining zygospores, it has been the subject of considerable investigation, as may be seen by reference to the literature cited under this species (p. 231). By Klebs ('98) the fungus could be made to produce zygospores on plain bread if the relative humidity in the surrounding air was sufficiently high. For Falck ('01), zygospores would form only when the concentration of the nutrient was sufficiently increased by the addition of certain solutions to the bread, and the relative humidity of the air seemed to be of slight importance.

Sporodinia is very common in this country on a great variety of Basidiomycetes of various groups. Dr. Farlow has been accustomed to use in his classes the zygospores of this species, and has sometimes obtained them from sowings on plain bread; and from all of several different collections the writer has been able to obtain an almost exclusive production of zygospores on the same substratum. A pure culture of this species was obtained in the summer of 1902 from material collected at Cold Spring Harbor, L. I., and has been kept running since in the laboratory. It has been noticed in general that in tube cultures sporangia have been exclusively present except in freshly made tubes

where there was presumably a greater humidity in the confined air and in which zygospores were also formed.

Further to test the effect of humidity on the type of fructification, a series of cultures was made from the material above mentioned under conditions which were made to correspond to those described by Falck in connection with his experiments. On March 17, discs of bread were sterilized dry in stender dishes, soaked, five of them with sterilized water and five with equal amounts of dilute prune decoction, and, after having been subjected for eight minutes to steam at 100° C., were inoculated with drops of water containing spore material. The stenders were left in a culture drawer until March 20, when a vegetative growth was first visible. One of the prune juice cultures showed young zygospores in process of formation, but in none of the other stenders had the growth advanced further than a production of the mycelium. The cultures were disposed according to the varying degrees of atmospheric moisture indicated in Table XI.

TABLE XI.

	Plain Bread.	Prune Bread.
Air very moist. Cultures open in moist chamber with caps of wet filter paper.	Only zygs.	Zygs. with tuft of sporangia.
Air normal. Cultures closed on laboratory table.	Only zygs.	Only zygs.
Air dry. Cultures open on laboratory table.	Only sporangia on surface, zygs. on sides below.	Only sporangia on surface, zygs. on sides below.
Air very dry. Cultures open in a sealed vessel containing calcium chloride.	Only sporangia.	Only sporangia on surface; zygs. and azygs. on sides below.

In general, the series demonstrates that increased relative moisture is a condition favoring formation of zygospores, while decreased relative moisture is a condition favoring the formation of sporangia. With the exception of the tuft of sporangia in the prune bread culture in very moist air, none of the cultures in normal, nor those in very moist air, produced any sporangia, while in the cultures in dry, and in very dry, air sporangia predominated. It was observed that the stenders in the

sealed vessel with calcium chloride suffered but little more desiccation than those exposed on the laboratory table.

From the fact that zygospores are readily produced on plain bread and from the observed effects of relative humidity on the type of fructification produced, the material investigated agrees with that used by Klebs, and the results obtained seem to give support to the suggestion that Falck worked with a form at least physiologically somewhat different, if not unusual.

Spinellus fusiger.

Next to Sporodinia, this species seems to have been more frequently found than any other homothallic form. It has not been cultivated by the writer, but he has been enabled to examine dried material from a number of different stations, as has been mentioned under this species in the Citations in Part I. The zygospores in all cases were found to be abundant between the gills of the host, and the connections of their suspensors could frequently be traced to the same branch of the spiny mycelium.

Its homothallic character is thus determined beyond question.

Mucors I and II.

This section will treat of a homothallic species or of a group of perhaps three forms which are very closely related. In October, 1901, the writer made a pure transfer from a Mucor growing apparently unaccompanied by zygospores on a culture of a decaying agaric and obtained the homothallic species which has been called in the present paper Mucor I. Mucor II was found by Professor Thaxter, January, 1898, at Daytona, Florida, growing spontaneously with its zygospores on a decaying Polyporus. A third form was obtained from a separation culture made in June, 1901, by Mr. E. E. Bogue from the "schleimflüss" on a recently cut birch stump. The mycelium was mixed on the soft agar with bacteria and yeasts, and produced on aerial hyphae only zygospores which were at first mistaken for sporangia. By making a number of mycelial transfers a pure culture was secured which produced sporangia as well as zygospores, and since this time no cultures have been obtained which would yield zygospores exclusively. In most cultures of this, as of the other two forms, zygospores and sporangia are generally both present in abundance.

It is possible that the form just mentioned is identical with one or the other of the first two forms. The three if not identical are sufficiently

alike for the purposes of the present paper to be treated as one, and though more cultures have been made with Mucor I the facts here presented will apply to them all. Mucors I and II are alike as far as their method of conjugation is concerned. They apparently disagree slightly, however, in their action in cultures and in their relations to temperature; and in cell cultures Mucor I, moreover, produces chlamydospores which bud in a characteristic Oedocephalum-like manner not possessed by Mucor II, but it is uncertain whether the slight differences which may exist are sufficient to separate these forms as distinct species.

The process of conjugation is similar to that determined in some detail in *Mucor Mucedo*. That there is a mutual attraction between the zygophoric hyphae is probable, and progametes which here as in all other cases always result from hyphal contacts, are frequently produced between the terminal portions of hyphae of such considerable length as to suggest that contact was brought about by the mutual orientation of the hyphae affected. In a few instances also in cell cultures hyphae have been observed growing toward each other and producing progametes at their point of contact. Not enough observations have been made, however, to demonstrate that this meeting may not be accidental. Attempts to follow the zygophoric hyphae is an uncertain task, since in cell cultures sporangial formation predominates and the production of zygospores cannot be limited to a single line, as can conveniently be done in the heterothallic forms. Moreover the zygophoric hyphae are not always readily distinguishable from the sporangiophores, and it has thus several times happened, during the course of an observation, that all the hyphae whose positions had been mapped and followed by the aid of a camera lucida have later been found to produce only sporangia. It can, however, be stated from observation that the contact of zygophoric hyphae is the stimulus for the outgrowth from them of the progametes, and that the latter never arise independently.

That the thallus of this plant is in fact bisexual has been shown by its response to both the sexual strains of a heterothallic species in the process of "hybridization" subsequently described (cf. p. 311 and Plate IV, Figure 56). No experiments have as yet been made, however, which will enable one to determine where the segregation of the sexes occurs before the formation of gametes. It is possible that any zygophoric hyphae may produce progametes when brought into contact with any other, and the segregation might then occur at the moment when the gametes were cut off. That such a process may occur is suggested by

the condition represented in Plate I, Figure 19, in which the zygophoric hyphae can be seen to have arisen in such a manner that there is an open protoplasmic connection between the two progametes, and in this respect the process is in marked contrast to that in Zygorhynchus, where the more delicate zygophore is always normally distinguished at the end of a filament by a septum below which the more vigorous zygophore arises as a branch. That the zygophoric hyphae may be primarily bisexual is suggested by the fact that, in one case observed, both of the zygophoric hyphae connecting with a zygospore had grown on to produce sporangia each of which we must assume contained bisexual spores. It is possible that the segregation may be partial in the zygophoric hyphae, giving to one a predominatingly (+) character and to the other, with which it forms progametes, a predominatingly (—) character. So far as they have been investigated, however, the mycelia developed from single spores are always equivalent as regards their sexual character. Separation cultures have been made both from transfers from single sporangia and from mixed transfers from a number of sporangia, and no difference could be observed in the zygosporic production of the different mycelial "colonies" developed, nor was there a line of greater zygosporic activity where the mycelia of two colonies came in contact. It is perhaps fruitless to speculate on these questions without more data than are at present available; but the writer hopes in the near future to be able to present additional experimental evidence bearing on the subject.

Homothallism appears to be a fixed condition in the species in which it has been found. During the six years Mucor II has been under cultivation in the laboratory, it has been continued to many generations of sporangial spores without suffering any abatement in its zygosporic activity, and the same is true, though to a less extent, of the other forms of the homothallic group. The thallic character, moreover, is not lost through the production or germination of zygospores, since although in water germinating zygospores produce only sporangia, in nutrients their germination gives rise to a mycelium from which are formed both zygospores and sporangia.

Since Mucor I was the first homothallic form that the writer had found and brought under cultivation, it has been the subject of a considerable number of cultural experiments, the object of which was to discover if possible the factors influencing the formation of zygospores in this species, as Klebs had done in Sporodinia, and to apply the results obtained in an attempt to secure zygospores in other forms. In these cultural investigations nearly two hundred pure gross cultures and some-

what over fifty cell cultures were made, but the production of zygospores was so little affected by the external conditions tested in the series which were conducted, that it seems undesirable to give more than a summary of the chiefly negative results obtained. In general, it may be said that zygospores were produced together with sporangia on all the substances tested, when the growth of the fungus was not obviously impeded.

Thus on such widely different substances as egg albumen, solution of peptone on sponge, solution of grape sugar on the same substance, and horse dung, zygospores were produced together with sporangia. Concentration of the available nutrient, in so far as tested, has practically no effect in determining the kind of fructification produced. On such dilute nutrient as could be procured from tap water with pure agar, or from sponge soaked with 0.5 per cent solution of grape sugar, with 0.5 per cent solution of peptone, or even with tap water, zygospores have been formed in small numbers along with a feeble development of sporangia. It sometimes has happened in the nutrients enumerated, that a few sporangia without zygospores were produced by the poorly developed mycelium. When spores are sown in such nutrient fluids as dilute prune decoction, an active fermentation takes place with an abundant liberation of bubbles of gas. Yeast-like forms develop, and although the mycelium generally remains submerged and septate, it may at times reach the surface and produce a scanty growth of sporangia sometimes accompanied by zygospores. Banana is the only substance upon which sporangia alone constantly form, and apparently this substratum is unfavorable to the growth of the fungus, for the sporangia are low in their development. In van Tieghem cells especially, when the substratum is disposed in a thin layer or tends to become dry, sporangia alone may be formed.

The relative humidity of the air seems within normal limits to be a comparatively unimportant factor in this case, and open cultures of favorable nutrients exposed to the air of the laboratory as well as those placed in a sealed vessel with calcium chloride have invariably formed zygospores below the luxuriant sporangial growth. Although zygospores seem to be somewhat more abundant when the cultures are exposed to a relatively moist air, desiccation, in the experiments tried, has not been able to check their formation to any considerable extent.

Temperatures up to at least 29° C. hinder the production of neither sporangia nor zygospores, and the difference between diffused light and absolute darkness is also without influence in this connection. Except in the separation culture from "schleimflüss," already referred to above,

no cultures have given an exclusive production of zygospores. In this instance, the soft agar used and the probable fact that the mycelium developed from a single spore may have especially favored zygospore-formation.

From the foregoing experiments it is evident that, in cases where the vegetation is interfered with, the formation of zygospores is apparently inhibited before that of the sporangia. In this connection it is interesting to note that, while in one of the forms obtained from soil by Hansen ('02) a similar relation existed between zygospore formation and unfavorable conditions at least of temperature, in another form from the same source the sporangia under similar conditions were found to be inhibited before the zygospores. In conclusion it may be said, in so far as concerns the three homothallic Mucors above discussed, that, in general, external conditions not obviously unfavorable to the growth of the fungus have no influence in determining which form of reproduction will occur

Zygorhynchus Moelleri.

This homothallic species, as already mentioned in the Citations in Part I (p. 227), has been found a number of times, the form with which the writer has experimented having been obtained at New Haven in a separation culture from soil by Professor Sturgis. It is similar in its mode of conjugation to *Z. heterogamus*, the other member of the genus which Vuillemin has separated from Mucor on account of the constant dissimilarity in the gametes. It may not be out of place in the present paper to give a brief account of the process of conjugation observed by the writer in *Z. Moelleri*, although in the main it will be but a repetition of the detailed description already given by Vuillemin in the other species.

In Mucors I and II, as has been seen, the zygophores generally arise from comparatively distant parts of the mycelium, and although their origin may be close together on the same mycelial filament, yet so far as has been observed zygospores are never formed between branches of a single aerial hypha. In Zygorhynchus, however, the conditions are reversed, and the two zygophoric branches almost invariably not only originate from a single aerial hypha, but have a definite position on the filament, and conditions in which zygospores have been observed to form between filaments connected with distant parts of the same mycelium are comparatively rare.

In the simpler case illustrated by the more common mode of conjuga-

tion, a terminal portion of an erect hypha is distinguished by a septum from the portion below. Immediately beneath this septum is produced a branch which, growing upward, recurves to meet the side of the slender zygophoric filament cut off by the septum already mentioned (Plate I, Figure 1). The two zygophores are from the beginning unlike in character as well as in origin. While the first, which contains but a small amount of protoplasm that becomes massed at the point of contact with the other, undergoes no further development, the second, which has arisen immediately below it, is from the outset richly supplied with dense protoplasm. Immediately after contact a progamete is developed as a perpendicular outgrowth from the slender erect zygophore, and in juxtaposition to this a progamete is formed by the terminal enlargement of the more vigorous zygophoric branch (Figures 2 and 3). In each of these progametes a transverse septum is formed, distinguishing the gametes which are unequal in size, the larger being formed on the side of the vegetatively more vigorous zygophore (Figures 4 and 5). This difference in size is always distinct, though in some cases (Figure 6), less marked than in others. The contents of the two gametes become united through the disappearance of the intervening wall, and the zygote here formed (Figure 7), by the gradual enlargement of the two cells thus united, assumes the shape of a mature zygospore (Figure 8). The supply of nutrient for this ripening process comes almost entirely by way of the more vigorous zygophoric branch, and, although the stretched wall of the larger gamete makes up the greater part of the outline of the zygospore, still the stretched wall of the smaller contributes to it. Although it may show a certain tendency in this direction, the condition here is thus not comparable to an oögamous fertilization where the male gamete furnishes protoplasm to, but forms itself no essential part of, the mature oöspore. There are neither cytological nor physiological data which will enable us to say with any degree of certainty which of these two unequal gametes is to be considered male in character, yet from the condition in oögamous Phycomycetes, it would be natural to assume that the male is represented by the smaller of the two uniting cells. The inequality in the size of the gametes is associated with a still greater inequality in their suspensors; while the suspensor subtending the larger is always swollen, a suspensor is seldom ever formed on the side of the smaller, since the septum which distinguishes the gamete just mentioned is usually directly continuous with the wall of the zygophoric filament. The mature zygospore thus appears to be borne terminally by the swollen suspensor and merely appressed against

the slender zygophoric filament. It sometimes happens that a short stalk remains after the smaller gamete is distinguished (Figure 4), and although it fails to enlarge and in the mature condition is hardly noticeable, its origin warrants the use of the term suspensor.

In some old cultures, cells have not infrequently been found in different stages of development, ranging from a condition in which the wall is only slightly discolored and papillate, to a condition with dark distinctly denticulate thick walls resembling, except in size, a mature zygospore (Figure 9). They are apparently formed from the smaller gamete developed from the less vigorous zygophoric filament, and on this supposition may be called azygospores. Though it is possible that azygospores are formed from the more vigorous zygophoric branches, such a condition has not been observed. It has not been possible to follow in detail their production, and to determine the immediate stimulus to their formation. The only explanation known for the origin of progametes is contact between the opposing zygophoric hyphae, and although no cases have been observed in which a contact could be considered responsible for the origin of the cells in question, their position on the filament and their general appearance suggests that they have been cut off from single progametes.

Although the species considered has been under cultivation for about ten years, and continued in pure cultures through many generations of non-sexual spores, no substratum nor external condition has been found which would prevent the formation of zygospores, notwithstanding the fact that all nutrients were tried for its cultivation which were used in the tests with Mucor I and II (p. 293) and Rhizopus (p. 248). The production of sporangia has been always less than that of zygospores, and it is often difficult to obtain preparations showing the former in any abundance.

In fluid nutrients like dilute decoction of prune, there is no fermentation nor production of yeast forms, and fructification only occurs when the mycelium reaches the surface, where it gives rise to a scanty growth of sporangia and zygospores. Nutrients of high concentration have not been used, but on such dilute nutrients as can be obtained from tap water in pure agar, or even from tap water alone on a sponge, zygospores and sporangia are formed together, although their production is feeble.

In the dryest atmospheres obtained, namely, in a sealed vessel with calcium chloride, zygospores are produced in abundance, as is also the case when the surrounding air is rendered nearly saturated by layers of

wet filter paper covering the culture. The maximum and minimum temperature limits have not been determined for the growth of the species, yet it is certain that both sporangia and zygospores are produced up to 29° C.

It may be seen from the foregoing cultural experiments that the formation of zygospores is practically independent of the external conditions to which the growth of the fungus is subjected.

Dicranophora sp.

This homothallic form, an undescribed species of the genus Dicranophora, has been given the writer by Professor Thaxter, who found it growing on Boleti at Kittery Point, Maine.

Few cultural experiments have been tried with the form, but such as have been made indicate that external conditions are more influential in determining the form of fructification than was the case in Mucor I and II and in Zygorhynchus. On the ordinary nutrient agar used in its cultivation, zygospores form abundantly on the substratum, and even in its interior, when soft agar is used. Upon tap water with pure agar growth is slow, and after several weeks the culture shows only a meagre production of sporangia without zygospores. Although when grown under favorable conditions of cultivation, sporangia and zygospores are formed simultaneously from the same mycelium, yet it is possible, by a decrease in the relative humidity of the surrounding air, to decrease the formation of zygospores and increase that of sporangia. Thus of three stender dish cultures on nutrient agar in which the mycelium had just become visible, the one left covered in a culture drawer produced abundant zygospores and comparatively few sporangia; while the other two, one of which was placed uncovered on a laboratory table, and the other in a sealed vessel with calcium chloride, where the surrounding air was comparatively dry, produced sporangiophores but no zygospores.

From the abundantly branched mycelium are developed numerous stubby, much-lobed branchlets the majority of which remain sterile. On account of the difficulty of distinguishing the sterile outgrowths from the parts destined to produce zygospores, and on account of the fact that the smaller zygophoric branch is usually beneath and concealed by the larger, it is a matter of some difficulty to obtain the early stages of conjugation. Enough has been seen, it is believed, to determine the main points of the process. The form is distinctly heterogamic, and a difference is from the very start apparent between the two zygophoric hy-

phae. They may both arise close together, with a proximal protoplasmic connection between them; and while one remains but little greater in diameter than the mycelial filament from which it arises, the other is distinctly swollen (Plate I, Figure 10). Stimulated by the contact, a bulge develops in the large zygophoric branch at its point of meeting with the other (Figures 10 and 11). When the gamete is distinguished from the slender branch, the bulge opposite is about equal in size, but in the case figured a septum has not yet formed distinguishing it as a gamete. Apparently a rapid increase in size takes place before this septum forms, and by the dissolution of the walls in contact an open communication is established between the two conjugating cells (Figure 12). At this stage of development the condition is essentially similar to that figured in Zygorhynchus (Figure 7). In the species under discussion, however, the smaller gamete forms a less proportionate part of the zygote than does the corresponding gamete in Zygorhynchus. The zygospore is developed almost entirely within the stretched and finally cutinized walls of the larger gamete, and a small beak which is generally present on the zygospore is the cutinized remnant of the wall of the smaller gamete. If the zygospore be crushed, a thick hyaline wall will be found to have formed within the boundaries of the original membrane of the zygote, and on its surface a protuberance of the same material of varying size may often be seen which apparently corresponds to the beak just mentioned (Figure 14).

Summary of Homothallic Forms.

The above five types of the homothallic group have been examined by the writer and, with the exception of Spinellus, all have been grown in pure cultures for several years. Although the figures and descriptions of many of the species reviewed under the Citations in Part I would indicate that these were also homothallic, yet in several instances, as in Phycomyces and Rhizopus, the account is shown by the results of the present paper to have been inaccurate as regards the thallic condition. The writer does not feel justified, therefore, in including in the homothallic group any forms, with the exception of *Zygorhynchus heterogamus*, which have not been examined with the distinct purpose of determining the thallic condition present.

If we compare the members of the homothallic group as thus limited, we shall find that they offer varying degrees of differentiation between the hyphae bearing zygospores and those bearing sporangia. In Spinellus, the zygospores are borne on a distinct spiny aerial mycelium.

In Sporodinia, although the aerial hyphae which bear sporangia and those which bear zygospores are somewhat alike in their method of branching, yet they are distinct, and each form of fructification is produced alone on individual filaments. In Dicranophora the zygospores are not aerial, but are formed between special short branches of the superficial or even slightly immersed mycelium. In Mucors I and II and Zygorhynchus, however, both zygospores and sporangia may be formed from the same aerial hyphae.

In so far as is known, external conditions have a marked influence on the kind of fructification produced only when the sporangia and zygospores are developed separately upon differentiated hyphae. Even in such forms as Sporodinia, in which external factors are most effective, under ordinary conditions zygospores and sporangia occur side by side, and it is only under extreme conditions that it is possible to obtain an exclusive production of either form.

The homothallic species thus exhibit a type of sexual reproduction which seems exceptional, and is distinctly different from that which is predominant in the Mucorineae as a whole; but although in several cases this process is not associated with any differentiation of the conjugative apparatus, such a differentiation is distinctly indicated in other instances, as has already been pointed out. This differentiation, the sexual nature of which can hardly be disputed, will be further discussed in connection with the phenomena of hybridization which afford further evidence in support of this view.

All the homothallic species studied have been kept under cultivation for a considerable time, — in the case of *Zygorhynchus Moelleri* for about ten years, — and no change in their zygosporic activity has been observed. No neutral strains of these forms, moreover, have been found, and homothallism therefore may be assumed to be a fixed condition in the forms in which it is known to occur.

It is a noticeable fact that no homothallic species have been found growing spontaneously on animal excrement, which is the most prolific source for forms of the Mucorineae. Sporodinia, Spinellus, and Dicranophora, in which alone a differentiation is apparent between the sporangiophores and zygophoric hyphae, have their natural habitat on the fructifications of certain fleshy fungi. Zygorhynchus has been found growing spontaneously on bread and on cultures from soil origin. The forms represented by Mucors I and II were obtained from Hymenomycetous fungi and from the schleimflüss of recently cut birch; and from Hansen's ('02) investigation it seems probable that soil is not an uncommon source of homothallic forms.

HYBRIDIZATION.

In the Introduction it has been already stated in some detail that there is a sexual response not only between the (+) and (−) strains of the same species but also between (+) and (−) strains of different species, and that no interaction ever occurs between strains with like signs. Although the process has not been observed to go further than a cutting off of the two gametes, and more frequently stops at the production of the two progametes or the formation of a gamete on but one side, the word "hybridization" has for convenience been applied to these imperfect attempts at conjugation. Just as, when the hyphae of two sexually opposite strains of the same species are allowed to come in contact, a dark line may be apparent by the accumulation of zygospores there formed (Plate IV), in a similar manner, when hyphae of sexually opposite strains of two different species are allowed to come in contact a white line may be apparent, which results from the accumulation of these attempts at hybridization above mentioned. Plate IV, Figure 57, is a photograph of a culture made to illustrate these lines of zygospores and hybrids. Where the (+) and (−) strains of Mucor V have come in contact, a very wide, dark line resulting from the zygospores there formed is apparent, while between the (+) growth of Mucor V and the (−) growth of a different heterothallic species, Mucor N, as well as between the (−) growth of Mucor V and the (+) growth of Mucor N, a white line resulting from the imperfect hybrids there formed is distinctly evident. The absence of such a white line between the growths with like signs results from the fact that no hybridization has occurred at their areas of contact.

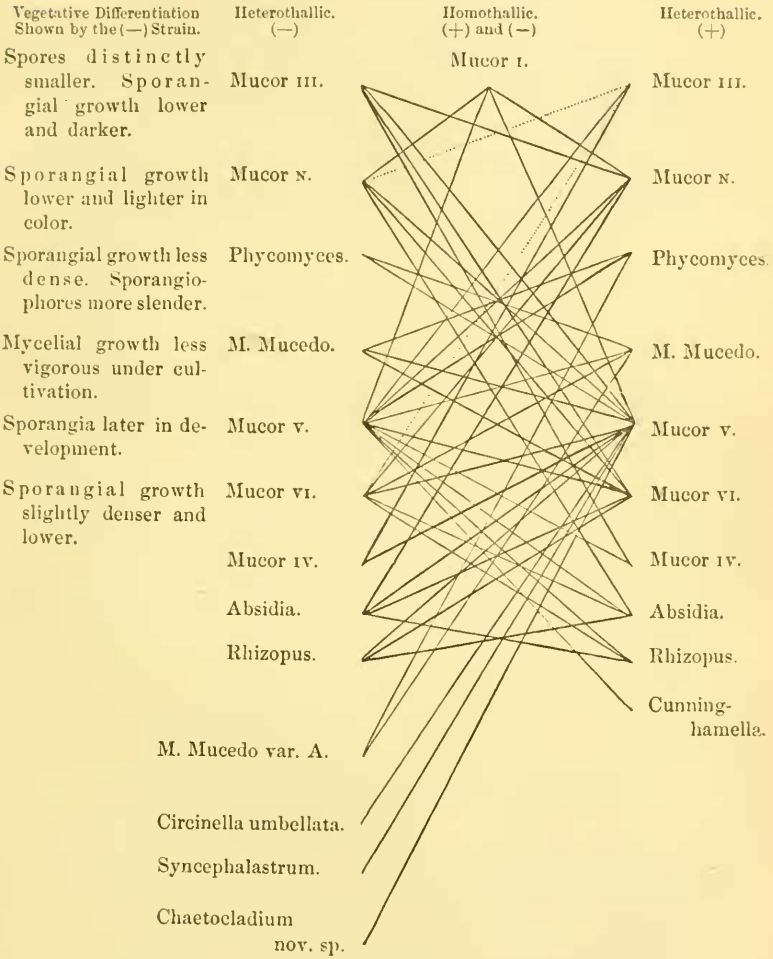
The first suggestion that an interaction is possible between sexual strains of different species arose during the investigation of the natural occurrence of sexual strains of *Mucor Mucedo*. A pure sporangial transfer from an unbranched Mucor growing on rabbit dung was inoculated into a stender dish together with inoculations of (+) and (−) strains of *M. Mucedo* so arranged that the mycelia in developing would come in contact with one another. In two days an unusually broad light line, apparently of young zygospores, resulted between the growth from this pure transfer and the (+) strain of *M. Mucedo*, and a similar line of less width occurred between the (+) and (−) strains of *M. Mucedo*. The day following the zygospores of *M. Mucedo* had become black in maturing, but the line between the unknown Mucor and the (+) strain of *M. Mucedo* remained white, and the conjugations

showed no further development than the production of progametes which in some cases had distinguished their gametes on one side. Pure cultures were made of this form which showed such a peculiar reaction, and labelled *M. Mucedo* var. A. Subsequent contrasts on a large variety of substances containing available nutriment in more or less concentrations showed that this arrest in the development of zygospores between var. A and the (+) strain of *M. Mucedo* was constant, and not due, as was at first thought possible, merely to the inability of the progametes, for some reason unusually abundant, to obtain from the substratum sufficient nourishment for their further development. Var. A in tube cultures is much slower and less luxuriant in growth, and is distinguished at once from *M. Mucedo* by the light yellow coloration of sporangia, hyphae, and mycelium. In addition there are certain slight structural differences which made it seem improbable that the form could be other than a distinct species, and suggested that the imperfect conjugations observed with the (+) strain might be considered analogous to a process of hybridization.

The opposite strains of the nine heterothallic species under cultivation, which represent five different genera, were accordingly contrasted with one another, and it was found that the opposite strains of every species are capable of hybridizing with strains of other forms. In this way it has been possible to arrange the opposite strains of all the different species in two series according to their interaction with one another. Mucor v has been found most active in the production of hybrids, and has consequently been the form chosen as a standard with which the other species have been tested. All strains that have been found to hybridize with the strain of this species, which for reasons to be shown presently has been called (+), have been placed together in Table XII in the column on the left, while those that hybridize with the strain called (−) have been placed in the column on the right. Continuous lines connect those strains between which hybridization has been accomplished, and dotted lines connect those strains between which attempts at hybridization have been unsuccessful. It will be noticed that in no case has the position of a strain in either column been determined by positive hybridization in tests with less than two other species. By an examination of the table it will be seen that in every instance when a differentiation is apparent in the opposite strains, the strain showing the less vegetative luxuriance has been located by the hybridization tests in the column on the left. In view of their less luxuriant character the strains in this column have been called (−), while those in the

TABLE XII.

THALLIC CONDITION OF FORMS INVESTIGATED AS DETERMINED BY HYBRIDIZATION.



other have been called (+). Before the possibility of correlating the opposite strains of different species was discovered, they were represented in cultures by arbitrary symbols, and though it seemed not impossible that the difference in strain luxuriance characteristic of certain

species was in all cases similarly connected with their sexual differentiation, there was no satisfactory basis for such a belief. It is now, however, hardly to be doubted that in the (+) and (—) columns are represented the two opposite sexes.

In testing the hybridization between two different species the four possible contrasts have in all cases been made. Generally the strains of one species have been successively inoculated in two slender cultures between the opposite strains of the other, but with spreading forms like Rhizopus and Absidia, in order to avoid the possibility of conjugation between the strains of the same species, it has been necessary to oppose but two strains in a single dish, and consequently in hybridizing such forms four slender cultures have been made. Although the presence of hybrids at the line of contact between (+) and (—) strains of different species is often indicated by a more or less distinct white line, this is not always the case, and with such forms as Rhizopus and Absidia no line is apparent, although a microscopic examination may show that hybridization has occurred. In every instance, whether a line has been visible or not, the growths at all four lines of contact have been microscopically examined before the position of the strains has been listed in the table.

In tests made between certain species no hybrids were obtained, but had the external conditions been altered it is possible that hybridization would have occurred. By changing the nutriment, hybrids were obtained, for example, between Phycomyces and *Mucor Mucedo*, but if, as sometimes happens, it is impossible to satisfy on the same substratum the conditions which are necessary in each of the two species for the formation of zygospores, no hybridization is to be expected when their strains are contrasted.

The difference in the abundance of hybrids and in the distinctness of the lines resulting between the sexually opposite strains of two species contrasted frequently shows a difference in the sexual stimuli exerted by the (+) and (—) strains of the individual species. In the most marked instance of this nature observed, a characteristic white line was formed between the (+) strain of Mucor N and the (—) strain of Mucor III, while between the (—) strain of Mucor N and the (+) strain of Mucor III on the same substratum, no hybrids were found, even though a microscopic examination was made of the region of contact where they might have been expected.

In the case of some species hybridization is more active with the (+) strain of Mucor V, while in other species the hybridization is more

active with its (—) strain. A difference in the sexual activity of the (+) and (—) strains of Mucor v thus seems apparent. If, however, instead of assuming, as has been done in the previous sentence, that the strains of all the other species are mutually equal in the strength of the sexual stimulus which they exert, we consider the strains of Mucor v as equal in this respect, it will be found that with some species the (+) strains and with others the (—) take the more active part in hybridization. This inequality in the sexual activity of the opposite strains in contrast between two given species has been constant on the limited variety of the substrata tested, but what significance should be attributed to it is not at present entirely clear. As will be later pointed out in the discussion of hybridization with homothallic forms (p. 311), the inequality in the lines of hybrids may be correlated with the sexual differentiation of the (+) and (—) strains.

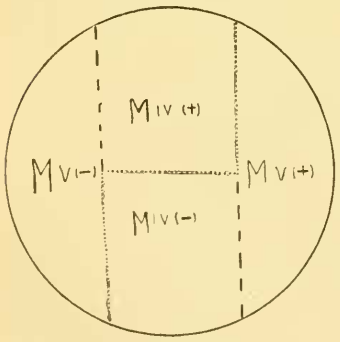
The difference which has just been under consideration is one between the individual strains of a single species, and can be seen only when hybridization is incited between two forms. Individual species, however, show varying intensities of sexual vigor, and while some can be induced to form zygospores only under special conditions, others will produce them under every condition to which they have as yet been subjected whenever their two strains are allowed to grow in contact. This condition is well illustrated by the fact that on certain substrata the production of the zygospores by (+) and (—) strains of Mucor iv does not occur, while distinct lines of hybrids are produced between strains of this species and of Mucor v respectively which are distinguished by unlike signs. Under somewhat different conditions of temperature and food supply contrasts of (+) and (—) strains of this form yield zygospores, but it is certainly remarkable that under any conditions the sexual response between them should be less intense than when they are contrasted against strains of a different species.

Mucor Mucedo var. A is the only form in which the process of hybridization has been followed on slide cultures. The formation of progametes with (+) strains of *M. Mucedo* appears to be essentially similar to the process where the contrast is made between the (+) and (—) strains of the latter species. In only a few instances have terminal cells been observed to have been cut off by septa in both progametes (Plate I, Figure 22). It frequently happens, however, that the gamete on one side only is distinguished (Figure 21), and a careful search in a number of slide cultures shows that, in the material examined, when only one gamete was formed, it invariably has been derived from the mycelium of

var. A. Whether a similar relation exists in other hybrids between the strains characterized by the greater development has not been determined. Var A has been grown in pure cultures and continued to the twentieth generation without suffering any apparent change in its growth or in its sexual activity.

Rhizopus and Absidia were the first species after *Mucor Mucedo* and var. A between which hybrids were obtained. On flour paste, which is a suitable substratum for the production of zygospores of Rhizopus but less favorable for those of Absidia, hybrids were meagre in development, and could be found only between Absidia (+) and Rhizopus (−). On potato agar, however, which is more favorable for the zygospores of Absidia but less favorable for those of Rhizopus, hybridization was more active. The commonest condition in the process of this hybridization is represented by Figures 16 and 17, Plate I. The hyphae belonging to Absidia can readily be distinguished from those of Rhizopus by the blue coloration of the former, but the difference in nature of the two progametes is strikingly apparent in the more advanced stages, where, as is shown in Figure 18, the characteristic outgrowths have arisen from the Absidia suspensor. In the instance figured a gamete has been developed on the Absidia side and septa have formed in either suspensor. The production of gametes on both sides, however, is rare.

That the sexual character in Rhizopus is not confined to special zygophores is confirmed by the fact that when the (+) strain, for example, of this species is contrasted with the (−) strain of Mucor v, the stolons of the former are thickly beset with hybrids which have arisen in response to the contact between hyphae of the sexually opposite strains. The hyphae of the Mucor recurve from all sides and apply themselves to the stolons of Rhizopus, presenting in cultures an appearance of yellow festoons radiating from the line of contact.



Mucor IV does not produce its zygospores at the room temperature of the laboratory on the nutrients ordinarily used for the cultivation of other forms. The accompanying diagram represents the condition in a stender dish culture of potato gelatine. Mucor IV (+) and Mucor IV (−) are contrasted with each other in the centre of the dish, and on the sides are contrasted

with Mucor v (+) and Mucor v (—). Broken lines represent areas of hybridization and dotted lines areas of contact between the different mycelia where no sexual response was apparent. It will be seen that lines of hybridization have occurred between the (+) and (—) strains of the two different species, but no zygospores have formed between those of Mucor iv. The strains of Mucor v seem to be characterized by an unusually marked activity in contrast to those of Mucor iv, which in this instance seem to play a comparatively passive part in the hybridization. In the warm oven and on a different substratum favorable to the interaction of the (+) and (—) strains of Mucor iv a similar arrangement of the same strains has yielded both zygospores and hybrids.

Mucor x will not produce zygospores when grown on such concentrated nutrients as are frequently used for the cultivation of other forms, and for this reason hybridization does not take place on these nutrients. With the (—) strain of Mucor v the line of hybrids is better developed than with the (+) strain of this species, and with the (—) strain of Mucor iii a line of hybrids is produced, while with its (+) strain no hybrids occur under the conditions present in the single test made.

Phycomyces nitens is unique among the heterothallic forms under cultivation in that its zygophoric hyphae are not necessarily aerial. For this reason it was thought that hybridization would not occur between it and other species with a different habit, and the first tests with *Mucor Mucedo* on potato agar by their negative results seemed to confirm this idea. On the substratum employed the two species will form their zygospores, but a higher concentration of nutriment than is afforded by such a substratum is more favorable to the production of those of Phycomyces. A nutrient agar made up with condensed milk has been found advantageous for the growth of this latter species, and was therefore used in subsequent tests, which gave positive results.

The response between the opposite strains of the two species when contrasted is shown by a light line at their areas of contact, but the process of hybridization has never been observed to go so far as a cutting off of gametes. With the (+) strain of *M. Mucedo* the response is more marked than with the (—) strain. The hyphae shown in Plate III, Figure 51, were taken from the line between the (—) strain of Phycomyces and the (+) strain of *M. Mucedo*. A filament of the latter is seen to be closely embraced by an irregularly lobed branch of Phycomyces and presents the appearance of being enclosed by the haustoria of some parasite. The hyphae of *M. Mucedo*, thus attacked by those of Phycomyces, seem to be but slightly modified themselves. They are more or less

branched, and, although generally sterile, cases have been observed in which sporangia have been produced from them. Sporangia, however, have never been found in direct connection with the convoluted hyphae of Phycomyces. The branches of *M. Mucedo* may be distinguished from those of Phycomyces by the fact that the walls of the former are covered with granulations, while those of the latter are smooth.

Mucor Mucedo does not as readily hybridize as some of the other forms; with Mucor VI, its sexual action is at best feeble, and often there is no response observable; and with Absidia and with Mucor III, although tests have been made, no hybrids have been obtained. The (+) strain of *M. Mucedo*, after having been rendered neutral towards its (—) strain by cultivation (p. 277), has been found no longer to form hybrids with var. A. This further emphasizes the conclusion that the stimulus for the production of progametes is the same in the formation both of hybrids and of zygospores.

As has been already mentioned, Mucor V is more active in forming hybrids than any of the other heterothallic forms known, and the strains of none of them have failed to respond when a contact with the proper strain of this species has been offered. For this reason Mucor V has been used in the few tests that have been made to determine the thallic condition of species the (+) and (—) strains of which have not been differentiated. They have been listed in Table XII (p. 305) under the sign opposite that of the strain to which they show a sexual response.

The zygospores of *Circinella umbellata* have been reported only by Bainier ('03), and in his figure he has represented a zygospore with its two suspensors in connection with the same hypha. Such a condition would indicate that the species is homothallic, but the evidence from hybridization points to a contrary conclusion.

Cultures of a new species of Chaetocladium, found by the writer in Venezuela, which can be readily grown saprophytically, and of Syncephalastrum, hybridize with Mucor V (+), and it is thereby rendered probable that these as well as some other species of the genus Mucor tested are individual (—) strains of heterothallic forms.

Cultures of *Cunninghamella echinulata*, a species originally described by Thaxter ('91) under Oedocephalum and recently redescribed as *C. Africana* by Matruchot ('03), who referred it to the Mucorineae largely on the ground that it acts as a host for Piptocephalis, have responded to the hybridization test with Mucor V (—), and have thus been shown to be (+) in character. In so far as may be determined, therefore, by the presence of a sexual response, the conclusion of Matruchot

in regard to the true position of the fungus appears to be thus substantiated.*

The species which we have been just discussing are either known or supposed to be heterothallic. An extended investigation of the reciprocal action of different species with the homothallic forms will be reserved for a later research. It has been determined, however, that, as was to be expected in the light of the present research, one at least from this group responds sexually to the (+) *and* (−) strains of heterothallic species. A demonstration of this nature is shown in Plate IV, Figure 56, which has been already described somewhat in detail in the Introduction. It is a photograph of a culture in which a homothallic species, Mucor I, was sown between the (+) and (−) strains respectively of Mucor V. The growth of Mucor I appears dark in the photograph on account of the abundance of zygospores produced from its mycelium. At the areas of contact between the growth of the homothallic species and that of either strain of the heterothallic species, a white line is visible, indicating the presence of hybrids. That hybridization thus occurs with both (+) and (−) strains, which may be assumed to be unisexual, shows that the thallus of the homothallic form investigated is bisexual, inasmuch as it produces hyphae which form progametes as a result of the stimulus of contact with *both* the sexually opposite strains tested.

When this same homothallic form is contrasted between the (+) and (−) strains of Mucor N, the lines of hybridization are less well marked and unequally distinct, that between Mucor N (+) and the homothallic species being much more evident. A similar but slighter difference between the lines of hybridization with the species previously mentioned can be seen in the photograph. It is also here apparent that the line on the side of the (+) strain is better developed. A like difference in the lines of hybridization has been already discussed under the hybridization of heterothallic forms. The most natural conclusion from these facts seems to be that the (+) and (−) characters in the bisexual mycelium of the homothallic species are equally active, and such an assumption would force us to regard the (+) and (−) strains of Mucor V and Mucor N as unequal in sexual vigor with the (+) the more active. These are the only two species which have been contrasted with a homothallic form, and therefore it is at present imprudent to state more than that it seems strongly probable that the difference in question is cor-

* Since this paper was in press the (−) strain of this species has also been obtained, and contrasts of the two yielded abundant zygospores July 27.

related with the difference previously observed in the vegetative vigor of the strains tested. If such be the case it will further emphasize the conclusion that the two opposite sexes are represented in the series containing respectively the vegetatively more and less vigorous strains.

By the facts presented in the foregoing section, it has been shown that a process of imperfect hybridization will occur between unlike strains of different heterothallic species in the same or even in different genera or between a homothallic form and both strains of a heterothallic species, and that, by taking advantage of this character, it has been possible to group together in two opposite series the strains of all the heterothallic species under cultivation. It has been seen, moreover, that when thus grouped the (—) or less luxuriant strains of all species known to show a vegetative difference between their opposite strains will be in one series, while the (+) or more luxuriant strains will be in the other. It has also been shown that when a contrast is made between the opposite strains of two different heterothallic species or between a homothallic form and both (+) and (—) strains of a heterothallic species the activity exhibited by these strains in the formation of hybrids at their lines of contact is generally unequal.

Hybridization may be assumed to indicate that the formation of progametes is determined by the stimulus of contact between the (+) and (—) hyphae whether of the same or of different species, and that the formation of zygospores is dependent on the union of gametes of the same species. From the facts observed it will be concluded that the formation of zygospores is a sexual process, that the mycelium of a homothallic species is bisexual, while the mycelium of a heterothallic species is unisexual, that differences in the hybridizing activity observed in opposite strains is correlated with the differences in vegetative activity which they exhibit, and further that in the (+) and (—) series of the heterothallic species are represented the two opposite sexes.

SUMMARY.

An outline of the general conclusions which have been reached as a result of the investigations embodied in the present paper has already been presented in the Introduction. A more extended discussion of the relations of the problems which have been suggested by the work of the past year the writer reserves until he has accumulated a greater body of facts on the subject. It now remains to give merely a brief recapitulation of the principal results considered in some detail in the foregoing pages.

(1) The production of zygospores in the Mucorineae is conditioned primarily by the inherent nature of the individual species and only secondarily by external factors.

(2) According to their method of zygospore formation, the Mucorineae may be divided into two main groups, which have been termed respectively homothallic and heterothallic.

(3) In the homothallic group, comprising the minority of the species, zygospores are developed from branches of the same thallus or mycelium, and can be obtained from the sowing of a single spore.

(4) In the heterothallic group, comprising probably a large majority of the species, zygospores are developed from branches which necessarily belong to thalli or mycelia diverse in character, and can never be obtained from the sowing of a single spore. Every heterothallic species is therefore an aggregate of two distinct strains, through the interaction of which zygospore production is brought about.

(5) These sexual strains in an individual species show in general a more or less marked differentiation in vegetative luxuriance, and the more and less luxuriant may be appropriately designated by the use of (+) and (−) signs respectively.

(6) In heterothallic species, strains have been found which from their failure to react with (+) and (−) strains of the same form have been called "neutral," and a similar neutrality may be induced by cultivation under adverse conditions.

(7) In all species of both groups in which the process of conjugation has been carefully followed the swollen portions (progametes) from which the gametes are cut off do not grow toward each other as currently believed, but arise from the stimulus of contact between more or less differentiated hyphae (zygophores), and are from the outset always normally adherent.

(8) In some species the zygophores have been demonstrated to be mutually attractive (zygotactic).

(9) In the heterogamic subdivision of the homothallic group a distinct and constant differentiation exists between the zygophoric hyphae and the gametes derived from them, but in the remaining homothallic forms and in all heterothallic forms no such differentiation is apparent.

(10) A process of imperfect hybridization will occur between unlike strains of different heterothallic species in the same or even in different genera, or between a homothallic form and both strains of a heterothallic species.

(11) By taking advantage of this character it has been possible to

group together in two opposite series the strains of all the heterothallic forms under cultivation.

(12) When thus grouped the (—) or less luxuriant strains will be in one series while the (+) or more luxuriant will be in the other.

(13) From the foregoing observations it may be concluded, —

(a) that the formation of zygospores is a sexual process ;

(b) that the mycelium of a homothallic species is bisexual ;

(c) while the mycelium of a heterothallic species is unisexual ;

(d) and further, that in the (+) and (—) series of the heterothallic group are represented the two sexes.

A preliminary summary of the results of the present paper has been given by the writer ('04) in Science, June 3, 1904.

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